Part I

INTRODUCTION

Photosynthesis is an essential and vital process for life on earth as we know it. The ability to convert the energy of solar radiation to chemical energy is the basis for a sustainable process that has enabled photosynthetic organisms to thrive for more than 3 billion years. They are essentially always at the bottom of the food chain. The ‘waste’ products of photosynthesis are molecular oxygen, coal and oil. Considering this impressive resume of photosynthesis, it is almost imperative to fully understand the underlying mechanisms and processes. In the near future our society will run out of fossil fuel reserves and we will be in dire need of an alternative and sustainable way to obtain energy. Sunlight is still the most promising energy source in terms of abundance, accessibility and sustainability. Out of these reasons and simply because it is a fascinating process, photosynthesis has been extensively studied for a long time. However, many questions remain and their complexity requires a broad interdisciplinary approach ranging from physics and biochemistry to molecular biology, plant physiology and ecology. The biophysical approach in this thesis focuses on the primary events of light-harvesting and excitation energy transfer in photosystem II (PSII) of plants. One of the main research questions concerns the regulation of a photoprotective mechanism called non-photochemical quenching (NPQ) that dissipates excessive excitation energy under strong light conditions. To that end we endeavoured to better understand the photo-physics of single photosynthetic complexes and investigated the effect of protein-lipid and protein-protein interactions via single-molecule fluorescence spectroscopy. The following general introduction is concluded by a short overview (section 1.11) of the research projects described in part II. A summary of the results and conclusions can be found at the end of this thesis.
INTRODUCTION

1.1 PHOTOSYNTHESIS

In plants, photosynthesis is commonly split into two separate processes called light-dependent (light) and light-independent (dark) reactions. The light reactions are the primary processes that convert the absorbed light into chemical energy. They take place in the thylakoid membrane of chloroplasts and the four main constituents are photosystem II (PSII), Cytochrome $b_6f$, photosystem I (PSI) and the ATP synthase \[ \text{[1]} \]. A model of the thylakoid membrane is shown in Fig. 1.1. PSI and PSII are tightly packed pigment-protein clusters, whose structures evolved to ensure that almost every absorbed photon can be utilized to drive charge separation. The photochemically active reaction centers (RCs) are surrounded by so called antenna complexes that increase the effective absorption cross section of individual RCs and provide enough energy for an optimum photochemical turnover rate. The second important function of the antenna system is a photoprotective mechanism called non-photochemical quenching (NPQ) that down-regulates the amount of available excitation energy under strong light conditions. NPQ is discussed in more details in section 1.6.

The negative charge of the primary pair resulting from photoinduced charge separation in the RC of PSII is transferred to a pheophytin and then quickly reduces a nearby quinone $Q_A$. The resulting semiquinone is further oxidized by a secondary quinone $Q_B$. The repetition of the same redox process results in a doubly reduced $Q_B$ that gets protonated and will in turn leave its binding pocket. The electrons from this plastoquinol ($QH_2$) are transferred by Cytochrome $b_6f$ to a water-soluble electron carrier plastocyanin (PC). This redox process pumps four protons from the outside Stroma to the inside Lumen of a chloroplast, resulting in a proton gradient across the thylakoid membrane. Additionally, for each electron entering the linear electron transport chain one proton is released into the Lumen via the water oxidation process. PC meanwhile re-reduces the primary donor of the charge separated state in PSI. The redox potential of the negative charge of the radical pair in PSI is eventually utilized to reduce NADP$^+$ to NADPH via Ferredoxin (Fd) and the ferredoxin-NADP-reductase (FNR).
The positively charged P680\(^+\) pigment radical in the RC of PSII has a very high oxidizing potential of \(E_m = +1.25\) V, which via an intermediate step involving tyrosine-Z is enough to oxidize water [2]. This water splitting process takes place in the oxygen-evolving complex (OEC) and results according to its name in the formation of molecular oxygen and protons that further increase the proton gradient across the thylakoid membrane. The ATP synthase utilizes this proton gradient to synthesize ATP.

In the dark reactions, referred to as the Calvin cycle, NADPH and ATP provide the chemical energy to drive the chemical synthesis of glucose. The reducing power created in the light phase of photosynthesis is used in several steps for CO\(_2\) assimilation [3].

### 1.2 Photosystem II

PSII is a large protein aggregate that consists of a dimeric core and the surrounding light-harvesting complexes (LHCs). A monomeric core complex of plants consists of about 20 protein subunits and contains 35 chlorophylls (Chls) \(a\), two pheophytins and 12 \(\beta\)-carotenes [4]. The peripheral antenna system of PSII is composed of monomeric LHC complexes called minors and multiple copies of a trimeric complex called major light-harvesting complex II (LHCII). The pigment composition of a monomeric LHCII complex entails 8 Chl \(a\), 6 Chl \(b\), 2 luteins (Luts), neoxanthin and one additional xanthophyll that depends on the xanthophyll cycle described later [5]. The minor LHCs are structurally
very similar to the LHCII monomer but do show differences in Car composition and Chl a/b ratio. The structural organization of PSII assemblies can be obtained from electron microscopy in combination with the individual crystal structures of the PSII core and the LHCs [5–8]. The largest PSII particle that has been obtained from biochemical purification is the so-called C2S2M2 supercomplex. It contains the dimeric core (C2), two strongly bound LHCII complexes (S-trimer) and two moderately bound LHCII complexes (M-trimer) [9, 10]. The minor LHC complexes CP24, CP26 and CP29 are present with two copies each per PSII dimer and mediate the binding of the larger LHCII trimers. Fig. 1.2 shows an illustration of a C2S2M2 supercomplex. The functional antenna size of PSII complexes in the native thylakoid membrane can be associated with up to eight LHCII trimers per supercomplex [9].

1.3 PIGMENTS: CHLs AND CARS

Pigments are the essential constituents of photosynthetic complexes that enable them to interact with light and harvest solar radiation. The main pigments in plants are Chls and Cars. Exemplary structures of the most common pigments are shown in Fig. 1.3. The tetrapyrrole ring of the chlorins Chl a and b with a centrally coordinated magnesium ion constitutes a conjugated π-electron system which gives rise to strong absorption properties in the visible electromag-
The absorption spectrum is characterized by strong absorption bands in the blue, often referred to as Soret bands, and two lower energy transitions called $Q_x$ and $Q_y$, referring to their polarization with respect to the pigment axes. The two pigments differ in one specific side group, a methyl group for Chl $a$ and an aldehyde group for Chl $b$, which results in a significant change of the absorption properties. One advantage of the heterogeneous absorption properties is the improved coverage of the solar radiation spectrum. The lowest site energy of Chl $b$ is generally above the lowest transition of Chl $a$ which is the reason that Chl $b$ is mostly found in antenna complexes while Chl $a$ is dominating in the core and RCs. This results in an intrinsic energy funnel, resulting in a higher probability for an excitation to reside close to the photochemically active site. The exact site energies vary from Chl to Chl due to different protein environments and due to varying degrees of excitonic coupling. Intra- and intermolecular vibrations interact with the electronic transitions resulting in a clearly observable Stokes shift in the fluorescence emission spectrum.

The fluorescence yield of unquenched Chls in a protein environment is about 30%. The remaining fraction of excited states decays non-radiatively via internal conversion (IC) and intersystem crossing (ISC) in about equal parts. The resulting large number of Chl triplet states can have an intrinsic excited state lifetime in the millisecond range. Furthermore, the transition energy is sufficient to convert molecular oxygen in its triplet state to singlet excited oxygen, which is highly reactive and might lead to the destruction of pigment-protein complexes. To prevent this detrimental process, additional Car pigments have the vital role to quench Chl triplets. The energy of essentially all Chl triplet is transferred to a nearby Car, resulting in an energetically lower Car triplet state that does not react with molecular oxygen. The Car triplet lifetime is only in the microsecond range, avoiding annihilation effects and temporary dark states as further explained in section 1.5. Cars furthermore contribute to absorption and extend the spectral range, e.g. Cars of plants feature strong absorption bands from about 400 to 520 nm.

1.4 **Excitation Energy Transfer**

A key feature in photosynthetic complexes is efficient excitation energy transfer (EET) from the location of absorption somewhere in the extensive pigment network to the RCs. The important factor is the amount of time it takes for an excitation to reach the RC and create a charge separated state. The rates for energy transfer and charge separation are in fact fast enough (when compared for
1.4 Excitation Energy Transfer

Figure 1.3: Structure of Chl $a$, Chl $b$, Lut and Violaxanthin.

example to the excited state lifetime of light-harvesting antennas in plants of about 3.5 ns) that more than 90% of excitation can be used for photochemistry. The energy transfer between pigments depends strongly on their distance and orientation due to the underlying dipole-dipole interaction. Moreover, antenna complexes are densely packed with Chl molecules in order to increase their absorption cross section. The equivalent density of Chl would in fact lead to significant concentration quenching of Chl in solution [17]. However, the specific arrangement and orientation of pigments within an LHC ensures fast inter-pigment energy transfer without a significant drop in excited state lifetime. The distance between nearest-neighbor pigments is often so small that their strong interaction has to be described with an excitonic model [18]. The complex excited state manifold and energy transfer rates within an LHCII monomer have been thoroughly investigated and modeled by a combined Redfield – Förster approach [19]. Intramolecular relaxation as well as EET from the Cars and Chl $b$ to Chl $a$ mostly happens on a subpicosecond time scale. Relaxation from bottleneck sites and intermonomeric EET shows transfer times up to 20 ps. The overall singlet excited state lifetime of isolated PSII supercomplexes increases with their size and is in the range of about 100 to 160 ps [20]. Studies on the trapping time of PSII in BBY particles [21] and thylakoids report values of about 150 ps and 300-400 ps, respectively [22–24]. The functional antenna size in these
membrane structures is larger though and the influence of size inhomogeneity and connectivity further complicate the interpretation of the obtained average lifetime.

### 1.5 Exciton Annihilation

One requirement of single-molecule spectroscopy (SMS) experiments is a sufficient signal to noise ratio of the detected fluorescence. This in turn requires a minimum photon absorption rate of the order of $10^5$ per second. At the necessary excitation power one has to consider the probability to have two electronic excitations present on pigments within one complex at the same time. The interaction of two excitations is usually referred to as exciton annihilation due to the effective dissipation of one excitation. This non-linear effect is commonly characterized and measured by a decrease in the fluorescence yield at increasing excitation powers.

There are mainly two types of annihilation: singlet–singlet (S–S) and singlet–triplet (S–T) annihilation [16]. As the name suggests, S–S annihilation refers to the dipole interaction of two singlet excited states. The excitation energy of one pigment is non-radiatively transferred to the second excited pigment, resulting in one pigment in the ground state and one in a higher excited state. The higher excited state quickly relaxes to the the first excited state via internal conversion (IC) (i.e. dissipation as heat). This annihilation process can be described by the following reaction scheme:

$$\text{Chl}^* + \text{Chl}^* \xrightarrow{\gamma_{SS}} \text{Chl}^* + \text{Chl}$$

The S–S annihilation rate $\gamma_{SS}$ is usually determined by the hopping or diffusion time of an electronic excitation and the probability of annihilation. The hopping time accounts for the influence of the domain size of multiple pigments connected via energy transfer.

However, S–S annihilation can be neglected in the experiments described in this thesis due to the high repetition rate of the laser. The pulse power is therefore so low that the probability of multiple excitations during one pulse can be neglected. Typical absorption rates of about one photon per microsecond are nevertheless on the same time scale as the microsecond lifetime of Car triplet states. The average population of Car triplet states depends therefore strongly on the average excitation power as explained in detail in chapter 2. The simultaneous presence of a Car triplet and an additional excited Chl singlet state results in S–T annihilation. The energy of an excited Chl singlet state is
Figure 1.4: Schematic of S–T annihilation. The singlet excitation (S\textsubscript{1}) on the left pigment (Chlorophyll 1) results with a yield of about 30\% in a Chl triplet state (T\textsubscript{1}) which subsequently gets quenched to form a triplet state (T\textsubscript{1}) on the Car. The singlet excited state of a second Chl (Chlorophyll 2) annihilates with the triplet state on the Car. Both Chls end up in the ground state while the Car triplet state remains in the first excited triplet state after rapid internal conversion from a higher excited state.

thereby nonradiatively transferred to a nearby excited Car triplet state. The higher excited Car triplet state then rapidly relaxes back to the first excited triplet state. The overall process effectively leads to energy dissipation of the Chl singlet excited state.

\[ Car^T + Chl^* \rightarrow^{\gamma_{ST}} Car^T + Chl \]

A schematic of this very similar process to S–S annihilation is shown in Fig. 1.4.

1.6 PHOTOPROTECTION

Carotenoids (Cars) play a major part in photoprotection as they quench Chl triplet states that would otherwise lead to the formation of harmful reactive oxygen species (ROS). However, this mechanism is only applicable in the antenna and not in the PSII RC because there is no Car close enough to quench Chl triplet states \[25\]. On the other hand, a high rate of photon absorption
can lead to a full reduction of the plastoquinone pool which in turn results in a high yield of Chl triplets in the RC due to the increased rate of charge recombination. An additional photoprotective mechanism called NPQ induces the dissipation of excessive excitation energy as heat and is therefore crucial to avoid photodamage [26–28]. This energy dissipation does not contribute to photochemical quenching and can be observed by a quenched fluorescence intensity, hence the name.

There are multiple components associated with NPQ and plants can furthermore acclimate to varying light conditions on different structural levels from protein composition and structural arrangement up to the chloroplast and leaf level [29]. One major part of NPQ that is rapidly reversible and triggered by the ΔpH across the thylakoid membrane is usually referred to as qE. The recovery phase of this quenching component takes a few minutes in the dark while the ΔpH recovers within 10 to 20 seconds. This difference in recovery time and other results that show the induction or sustained presence of qE without a ΔpH indicate that the pH gradient is only the trigger and does not in itself constitute the molecular mechanism associated with qE.

Another important component of qE is the protein PsbS that is required for NPQ in vivo. It is widely assumed that PsbS facilitates the formation of qE by making the photoprotective response more sensitive to native levels of ΔpH [30, 31]. High enough levels of ΔpH can indeed still induce qE without the presence of PsbS [26]. Another study showed that in the absence of PsbS qE is still triggered, albeit on a much slower timescale [32]. The location of PsbS is not entirely clarified but it is thought to bind to several Lhcb proteins and is likely interacting with LHCII. PsbS can form dimers that are more stable at low pH and it either increases the sensitivity to protonation or directly influences the conformational state of LHCII [33].

It has also been shown that the pigment zeaxanthin and the xanthophyll cycle in general are closely related to NPQ. The formation of zeaxanthin by violaxanthin de-epoxidation is directly linked to a lowered pH and the presence of zeaxanthin within LHCII can explain its response to the ΔpH. Although zeaxanthin has been proposed to be a direct quencher in NPQ [34, 35], other results point more towards a regulatory and allosteric role similar to PsbS.

Aggregation of LHC complexes is another common hypothesis to explain the underlying mechanism of NPQ. Aggregation in vitro indeed results in significant amounts of fluorescence quenching sufficient to explain NPQ. Also, both PsbS and zeaxanthin have been hypothesized to regulate aggregation [36].

The most promising explanation for the molecular mechanism of quenching is the formation of a non-radiative decay channel via Lut. Small conformational
changes resulting from protonation or aggregation can change the interaction between Lut and nearby Chl a and open up an effective energy dissipation channel [37, 38].
1.7 SINGLE-MOLECULE SPECTROSCOPY

The content of this thesis is largely based on single-molecule spectroscopy (SMS) experiments performed on a home-built confocal fluorescence microscope. A significant part of this thesis entailed the development of the setup to allow time-resolved spectroscopy in the time range of picoseconds to milliseconds. Time correlated single photon counting (TCSPC) is a time-domain fluorescence lifetime technique that has been combined with SMS to analyze the fluorescence decay and therefore the intrinsic photo-physics of individual fluorescence particles. To access the time range of micro- and milliseconds I implemented a modulated laser excitation technique by utilizing an acousto-optic modulator. Together, these two techniques facilitated the identification and analysis of quenching mechanisms in individual photosynthetic complexes.

The beauty of single-molecule experiments is the fact that ensemble averaging, typically observed in bulk measurements, can be avoided. This allows to measure distributions instead of average values which enables for example the identification of sub-populations in a heterogeneous sample. The width of a distribution and the spectroscopic comparison of bulk and single-molecule data further help to study static and dynamic properties of individual particles on various time scales. The direct observation of state changes in individual particles is another advantage of SMS as it does not require synchronized state changes of multiple molecules. In order to detect these dynamic changes it is advantageous to measure the fluorescence of one individual complex for as long as possible. This can be achieved by immobilizing the sample on a substrate, actively tracking and following a single particle or forcing it to stay in the desired location. The latter is for example realized in the Anti-Brownian electrokinetic trap (ABEL trap) [39]. We coated cover glasses with a positively charged mono-layer of poly-L-Lysine (PLL) to electrostatically immobilize the overall negatively charged complexes on the surface. The density of surface bound complexes is adjusted empirically to resolve individual complexes and to minimize the probability to have multiple units in the confocal spot of the microscope.

Cryogenic SMS experiments on LHCs directly visualize the energy of individual pigments and excitonic states due to spectral narrowing. Often the vibrational substructure can be obtained as well and the overall fluorescence spectra help to resolve energy transfer dynamics, spectral diffusion and the degree of disorder[40].

At ambient temperatures, the electronic structure cannot be determined as accurately due to spectral broadening. The conditions are nevertheless much
closer to the native environment and the measured fluorescence dynamics can be interpreted from a functional point of view.

A schematic of the used confocal fluorescence setup is shown in Fig. 1.5. The Titanium:Sapphire laser produces 200 fs pulses at 830 nm. The subsequent optical parametric oscillator is aligned to produce 633 nm pulses. An acousto-optic modulator can be used to modulate the excitation pattern in a time range from less than a microsecond to seconds as explained in section 1.10. Near-circularly polarized light is achieved by a berek polarizing compensator in order to avoid selective excitation due to the orientation of immobilized complexes. A refocusing unit checks and corrects the distance of the sample surface to the objective. The collected fluorescence light can either be detected with a single-photon avalanche photo diode to investigate the time-resolved fluorescence intensity and fluorescence lifetime (TCSPC) or is dispersed on a grating to measure the fluorescence spectrum with a CCD camera.

1.8 FLUORESCENCE INTERMITTENCY ("BLINKING")

A prominent feature that is associated with single emitters is fluorescence intermittency, often referred to as fluorescence blinking. These rapid fluctuations in fluorescence intensity between a bright fluorescent (unquenched) and a dark
state can be observed in a broad range of fluorescent systems, e.g. organic molecules, dyes, quantum dots and fluorescent proteins. These states are usually referred to as on-state (fluorescent) and off-state (dark). In contrast to single molecules that indeed switch between a fluorescent and a completely dark state, more complex systems often exhibit intermediate states that are not completely dark or only partially quenched. These intermediate states nevertheless also emit at stable fluorescence intensity levels with dwell-times from microseconds up to seconds or even minutes. An intriguing property of blinking is indeed the broad range of switching rates, often spanning more than five orders of magnitude in time. The dwell-time distribution of these various fluorescent states often follows a power-law behavior, sometimes adjusted by an exponential cut-off function [41]. Although this blinking phenomenon is similar for a wide range of systems, the physical origin is still under debate and most likely varies for different systems. One common hypothesis is the influence of the environment on blinking statistics, e.g. blinking in quantum dots can be controlled by core-shell engineering that reduces Auger-recombination on the surface of the quantum dot core [42]. Photostabilization in fluorescence chromophores by solution additives or by covalently linked photostabilizers that drastically reduce blinking is another example for the influence of the environment [43, 44]. Reducing or stopping fluorescence blinking in fluorescence reporters is important to improve the fluorescence yield and stability in fluorescence imaging and spectroscopy applications. However, recent advances in super-resolution microscopy actually utilize the statistical nature of blinking together with localization methods to achieve high resolution images [45].

LHCs exhibit significant blinking as well and the question arises whether it has a functional role in native light-harvesting. It has been shown in single LHCII complexes that fluorescence blinking is indeed sensitive to environmental changes, i.e. lowering the pH and removing the detergent increases the occurrence and dwell-time of complexes in a quenched state [46]. As fluorescence spectral dynamics in single LHCs can be explained by small conformational changes of the pigment-protein complex [47], conformational changes are also the most likely explanation for fluorescence blinking [48]. Blinking events will further directly influence the efficiency of energy transfer to the RCs due to an increased rate of energy dissipation in the quenched domain of a PSII supercomplex. It is therefore very interesting to investigate the involvement of fluorescence blinking in qE, which was explained in section 1.6.
1.9 Fluorescence Lifetime Measurements and TCSPC

The fluorescence lifetime of a fluorescent particle conveys information on the overall excited state decay kinetics. But it indirectly also contains information on energy transfer kinetics, connectivity and structure. Contrary to the steady state fluorescence intensity, the fluorescence lifetime allows to distinguish dynamic from static quenching, i.e. whether the non-radiative or radiative decay rates change compared to bleaching or non-equilibrium quenching effects. The two main ways to measure the time-resolved fluorescence are time-domain and frequency-domain methods. A detailed general description of these methods can be found in [49]. For our setup we made use of the existing pulsed laser and added the required fast electronics to perform time correlated single photon counting (TCSPC) experiments. The general principle of this time-domain method is to measure the time delay between a detected fluorescence photon and a periodic reference signal which is conveniently taken from the pulsed laser source. The optical pathlength of excitation and detection light as well as the time delay of electronic components only add a constant time delay that can easily be accounted for. The measured relative time delay depends on the time a fluorophore remains in the excited state before it eventually decays via fluorescence. A single photon detection event does not yield much information but measuring multiple time delays allows to build up a histogram. This fluorescence decay histogram $N(t)$ then corresponds to the time-dependent probability of the fluorophore to be in the excited state $I(t)$, convolved with the so-called instrument response function $IRF(t)$ of the setup.

$$N(t) = (I * IRF)(t) = \int_0^t IRF(t - t') I(t') dt'$$

The IRF constitutes the sum of timing errors of the whole setup including the pulse width of the excitation laser, time resolution of the detector and timing error of the electronics. In our case the time resolution of the detector is the dominating error compared to the laser pulse width of about 200 fs and the timing error of the electronics of less than 12 ps (RMS). The IRF can be measured experimentally by using a dilute scattering solution or the reflected light from the surface interface, effectively providing a zero-lifetime sample. Fig. 1.6 shows the measured IRF and its FWHM at different detection wavelengths (detector: Micro Photon Devices, PDM series, 20 μm diameter of active area).

The electronics to measure the time delay are based in our PicoQuant system on a time to digital converter (TDC) that registers the time for both the laser
trigger and the fluorescence photon detection with respect to a precise internal crystal clock. Each input channel is equipped with a constant fraction discriminator (CFD) to determine the arrival time of the voltage signals coming from the fast photo diode of the laser and the single-photon avalanche diodes (SPADs) for fluorescence detection. Apart from the relative time delay, this technology also allows to register the absolute time of TDC events with respect to the start of the measurement, so called Time-Tagged Time-Resolved (TTTR) data. Constructing a fluorescence intensity time trace is a straightforward application of this absolute timing information. One advantage is that the binning time can be adjusted after the measurement due to the precise timing of each detected photon.

In order to evaluate the performance of the TCSPC setup, we measured the fluorescence decay of pinacyanol in ethanol and methanol and extracted the fluorescence lifetimes (See Fig. 1.7). A reconvolution fit based on the measured IRF allows to account for the broadening effect of timing errors of the instrument. As a result, lifetimes slightly smaller than the FWHM of the IRF can resolved. The obtained lifetime value for pinacyanol in methanol of about $7 \pm 5$ ps (SD of five individual measurements) is well within the range of previously published values and close to the 6 ps recently measured with a streak camera setup [50]. Pinacyanol in ethanol has a slightly longer lifetime and the measured value of $16 \pm 4$ ps is also close to the previously reported value of 13 ps [51].
1.10 Modulated Excitation: Resolving μs and ms Kinetics

The pile-up effect, i.e. lost photon detection events due to the dead time of the TCSPC device, can be neglected in typical SMS experiments due to the intrinsic low detection rates of less than $10^4$ cps which is lower than 1% of the repetition rate of the excitation laser.

The time resolution of fluorescence intensity time traces is due to the absolute signal detection rate of the order of $10^3$ cps and SNR considerations limited to minimum binning times on the order of 10 ms. However, in order to characterize the photophysics and conformational dynamics of LHCs one needs to cover a large time domain, especially also the micro- and millisecond time scale. TCSPC helps to resolve the photophysics in the picosecond and nanosecond time range but ordinary TCSPC histograms are limited to the time range between two subsequent laser pulses. The MIRA laser system has a repetition rate of 76 MHz which corresponds to an accessible time window of 13.16 ns. Pulse picking gives a possibility to access longer timescales up to milliseconds but the required peak pulse power to achieve the same average fluorescence count rates would be significantly higher. We chose instead to utilize an acousto-optic modulator (AOM) to modulate the excitation power. Triggering the AOM with
Figure 1.8: This scheme illustrates the modulated excitation technique to measure changes in the fluorescence yield on the microsecond and millisecond time scale. The gray area illustrates the modulation of the laser excitation by utilizing an acousto-optic modulator (AOM) as a binary shutter. The red lines indicate the underlying individual laser pulses with a frequency of 76 MHz. The time tagged arrival time of fluorescence photons can be used to build up a fluorescence lifetime histogram as indicated in the bottom right panel. The two dashed lines indicate two exemplary fluorescence decays. These histograms correspond to the TCSPC technique described in the previous chapter. The arrival time of the same detection events in relation to the AOM modulation can also be used to build up an AOM histogram that in an analogous way illustrates fluorescence kinetics on the time scale of the AOM modulation. This is shown in the bottom left panel. The decreasing dashed line illustrates an exemplary situation where the fluorescence yield decreases during the on-time of the AOM modulation and the detected photons are bunched at early AOM delay times. If there are no changes in the fluorescence yield, the histogram will be flat. Two exemplary detection events are shown as black stars. The two black bars in the two lower panels indicate the different histogram positions of the same two photon detection events (black stars) in the AOM histogram compared to the fluorescence lifetime decay. The dashed blue line illustrates the evolution of the triplet state population as an exemplary application. The fluorescence intensity in the AOM histogram decreases due to the increase in probability to generate a triplet state in the system.
pulse wave forms from a National Instruments (NI) Timing card enabled us to use the AOM effectively as a fast shutter. The on and off times can be controlled by the NI card and the accessible time range covers intervals above about 50 ns. Histogramming the arrival time of fluorescence photons (TTTR data) over one on–off cycle of the excitation modulation results in a fluorescence intensity histogram that directly visualizes changes in the fluorescence yield during the modulation cycle. The AOM modulation method itself does not require a pulsed laser as long as the modulation of the excitation intensity can be time gated in respect to the detection of fluorescence photons. However, utilizing a pulsed laser allows one to investigate the change in fluorescence lifetime (TCSPC) as a second dimension during the AOM modulation interval. The signal to noise ratio of the fluorescence signal is crucial as this technique is based on the measured changes in fluorescence intensity due to changes in the fluorescence yield.

This technique is applied and further explained in chapters 2 and 6 and exemplary histograms are depicted in Figs. 2.11 and 6.6.

1.11 THESIS OVERVIEW

The goal of this thesis is to investigate and better understand the photophysics of individual light-harvesting complexes of PSII in experimental systems and environments that come as close as possible to the native thylakoid membrane. Single-molecule spectroscopy is the utilized experimental technique, suitable to measure the fluorescence intensity and spectrum of single particles. The existing confocal fluorescence setup was first extended by a state-of-the-art technique (see section 1.9 on TCSPC) to measure the fluorescence lifetime of single complexes. Information about the fluorescence decay is essential to study excited state dynamics and energy transfer kinetics. This time-resolved technique to measure relative and absolute delay-times of fluorescence photons was further developed to access fluorescence kinetics on the micro- and millisecond time scale as described in section 1.10.

Before introducing changes to the experimental system it is important to carefully characterize the fluorescence decay of single LHCs in commonly used and well studied detergent micelles. It turns out that the fluorescence decay of LHC complexes is significantly influenced by S–T annihilation, at least and especially at excitation intensities used for SMS experiments. Chapter 2 gives a detailed explanation in what way and how much this effect changes the overall decay of electronic excitations within trimeric LHCII complexes. The experimental results and complimentary mathematical modeling allowed us to successfully
apply and validate a stochastic model for S–T annihilation that can be more generally applied to describe small molecular aggregates containing multiple pigments.

Chapter 3 is based on the results from the previous chapter and compares the fluorescence characteristics of LHCII trimers in detergent micelles with trimeric LHCII in lipid nanodisks. It discusses the obtained sample from a recently developed protocol to directly isolate photosynthetic complexes in a lipid environment. The main questions concern the sample quality, stability in terms of photodamage and influence of the lipid environment on the excited state dynamics.

The following chapter 4 describes a different approach to investigate a lipid environment by incorporating LHC complexes into lipid vesicles (liposomes). Apart from studying the influence of lipids on the photophysics of a single LHC complex in itself, another focus lies on the interaction between multiple LHC complexes within a single liposome. How, both qualitatively and quantitatively, does crowding or clustering of LHC complexes influence the fluorescence decay and therefore the intrinsic capacity to deliver excitations to the photochemically active RC? The experimental results furthermore provide surprising but very interesting insights into the structural integrity of LHCII complexes within liposomes.

The content of chapter 5 is a slight detour into the more fundamental electronic properties of the pigments contained in a monomeric LHCII complex. Comparing the spectral properties of wild-type LHCII with a reconstituted mutant that lacks Chls \(a611\) and \(a612\) of the excitonically coupled Chl cluster \(a610-a611-a612\) provides a possibility to investigate the conformational flexibility and functional role of this terminal emitter domain. A combination of bulk and single-molecule spectroscopy together with mathematical modeling results in a conclusive explanation for the observed spectral properties of the mutant and allows us to formulate a design principle for the wild-type pigment configuration in terms of robustness and energy transfer efficiency.

In an attempt to approach a more native and functional assembly of PSII, the final chapter 6 describes the time-resolved fluorescence kinetics from picoseconds to seconds in single PSII supercomplexes. The main questions concern the identification and characterization of intact PSII supercomplexes and the explanation for the surprisingly fast fluorescence decay. The obtained light-dependent fluorescence kinetics are discussed in the context of fluorescence blinking and NPQ (see sections 1.8 and 1.6).