

Determining the Energy Pathways in Light Harvesting Complex II using Femtosecond Laser Techniques at Two Excitation Wavelengths

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Abstract

The transfer of energy in the photosynthetic process is extremely fast with transfer times extending between tens of femtoseconds to a couple of nanoseconds. The main light harvesting complex in plants is LHCII and it is the energy transfers between the molecules in this complex that will be explored in this project. Due to the fast dynamics of these transfers, femtosecond pump-probe spectroscopy was employed to determine the energy decay pathways taken in the LHCII (extracted and isolated from the leaves of spinach and acidphila) upon the excitation of the carotenoid region at wavelengths 494nm and 520nm. Glotaran was used to perform a global analysis on the two datasets, with sequential decay, to model the difference absorption spectrum at various times and four lifetimes were resolved from the data. It was found that, in the first resolvable lifetime, the ground state bleach that occurred at the Q_y state of Chla and Chlb on a sub-picosecond scale (670fs and 710fs for the first and second data set respectively) was due to internal conversion from their corresponding Soret band. The energy transferred from both Chlb and the Cars to Chla occurred within a few picoseconds (5.135ps and 7.66ps respectively) and after a couple of nanoseconds the depletion of excited states in the Cars and Chlb was evident having transferred their excitation energy to Chla. At this final lifetime fluorescence of Chla was observed in both datasets between 2.7656ns and 6.897ns.

0.1 Abbreviations

PSII - Photosystem II
LHCII - Light Harvesting Complex of photosystem II
Chla - Chlorophyll a
Chlb - Chlorophyll b
Cars - Carotenoids
Lut - Lutein pigment
Neo - Neoxanthin
Vio - Violaxanthin
IC - Internal Conversion
ISC - Intersystem Crossing
GSB - Ground State Bleach
SE - Stimulated Emission
ESA - Excited State Absorption
EET - Excitation Energy Transfer
OD - Optical Density
NOPA - Noncollinear Optical Parametric Amplifier

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Chapter 1

Introduction

1.1 The Nature of light

Light exists in quanta of energy called photons that have particle-wave duality and behave according to the theory of Electromagnetism. Due to the wave like nature of light, the parameters of frequency (f), speed of light (c), and wavelength (λ) are related by:

$$c = \lambda f$$

and due to the particle nature of light the wavelength of the photon can also be related to its energy (E) by the Planck-Einstein equation:

$$E = hf$$
$$= \frac{hc}{\lambda} \tag{1.1}$$

where h is Planck's constant. Equation 1.1 is the basis of what takes place in the mechanism of photosynthesis and thus is foundational for this study.

1.2 Primary Processes of photosynthesis

Photosynthesis is a process whereby energy, in the form of light, is converted to chemical energy in higher order plants, cyanobacteria and other bacteria with photosynthetic capabilities. These are the only organisms that can achieve this type of energy conversion. The key component to the primary process are the photon-absorbing pigments, bound to proteins, which make up a light harvesting antenna system of the plant [1][2][3]. A pigment is a chemical compound that absorbs light at certain wavelengths and reflects at others. Energy from the light that is absorbed by these pigments or a group of pigments is transferred to the reaction centre where it is then converted into chemical energy. The collection of pigment-protein macromolecules form individual photosystems known as photosystem I and II (PSI and PSII respectively). These are located in the Thylakoid membrane of the leaf (see Figure 1.1) which are built together to form grana stacks which make up multiple layers in the chloroplast of the leaves where all the light induced reactions take place. The photosystems are comprised of light harvesting complexes, such as

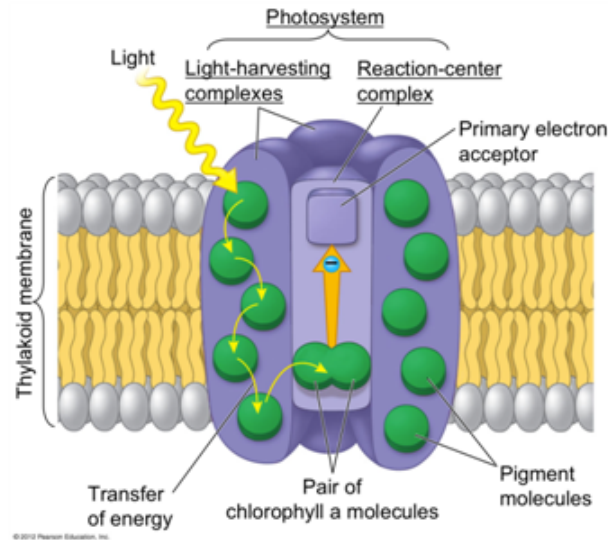


Figure 1.1: An animated depiction of the photosystem in the Thylakoid membrane of the leaf to illustrate. [4]

LHCII in photosystem II, among others. In addition to these, the photosystems contain other monomeric pigments and a core which contains the reaction centre. Photosystem II will be explored in section 1.4. The two photosystems, although very similar, absorb at slightly different frequencies since they are comprised of a few different pigments.

Light incident on the leaves of plants results in the excitation of the light harvesting pigments and after a series of energy transfers the excitation energy is transported to the reaction centre where it excites special trap chlorophylls. At this point the excitation energy received by these results in a photochemical reaction whereby the excited electron is removed from the chlorophyll molecule and given to an acceptor. It is then replaced by an electron from a donor molecule. In this way there is a flow of electrons that start

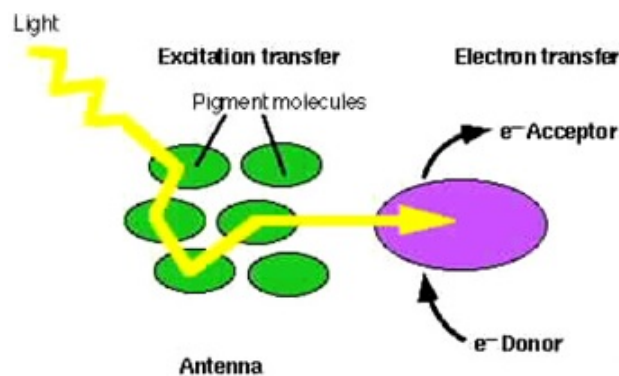


Figure 1.2: A simplified diagram of the energy transfer between pigments on the way to the reaction centre. [5]

the transport chain to the NADP unit of the photosynthetic system which will not be considered here. This is summarized in Figure 1.2.

The orientations of the pigments and the way they are packed together is crucial for maximum efficiency of these energy transfers so that minimal energy is lost between

transfers. The rate of energy transfer is extremely fast, ranging from less than 100 fs to a few hundred picoseconds so in order to study the energy transfers that take place in photosynthesis, ultrafast methods are required (where ultrafast refers to any time in the range from 100 femtoseconds to a few picoseconds). The method that was implemented in order to study the energy dynamics in LHCII of PSII was that of ultrafast pump-probe spectroscopy with a femtosecond laser setup.

The aim of this project was to understand and describe the ultrafast energy dynamics that take place in between the carotenoids (other light harvesting pigments) and the chlorophylls in real time just as they would occur in the biological environment of the specific antennae complex, LHCII, and compare these findings with those in literature. Another purpose was to become familiar with the experimental procedure and methods that are used in biophysics studies in the area of photosynthesis and molecular biophysics.

1.3 Molecular absorption and orbital theory

As the system under consideration (LHCII) consists of many molecules interacting with one another, a brief introduction to molecular orbital theory is required. A molecule contains some electrons that are kept to the individual atoms of the molecule (localized electrons) but also electrons that are shared between nuclei in the molecule (delocalized electrons). The probability distribution of the molecule informs us of where these electrons are most likely to be located and is found by taking the linear combination of the atomic probability distributions of the molecular constituents.

The term ‘ground state’ for a molecule refers to the state in which the nuclei are in the equilibrium positions with no net force acting on each one from other nuclei and electrons [6]. The electrons all have ground state energy and are in their original molecular orbitals.

There are two ways in which a molecule can be raised to a higher energy level. Either due to electromagnetic radiation whereby a molecule absorbs a photon or the molecule can be raised to a more energetic state by interaction with another molecule (vibrational interactions or the transferring of an excited electron to the other molecule). The energy of the incoming radiation determines to which excited state the molecule is raised to.

The Stark-Einstein law states that the absorption of one photon results in the excitation of only one molecule [7]. Einstein went a step further to say that each photon excites a single electron in that molecule. When the electrons are oscillating back and forth in the potential well, their probability distribution is dependent on their vibrational state. The electron is most likely to be found at the turning points of its oscillation as its vibrational energy increases. In an atom this is a straight forward simple harmonic motion problem but in a molecule where there is more than one nuclei, that are themselves oscillating, this becomes a more complex system. The Franck-Condon principle (illustrated in Figure 1.7) can be applied here whereby the nuclei, being heavier than the electrons, is taken to move much more slowly in transitions between electronic states. So much so, that the nuclei can be taken to be stationary during the rapid transition of the molecule to an excited state due to the photon. This means that the transitions between energy levels can be depicted as vertical lines due to the fixed nuclear frame. The same principle applies in the case of emission.

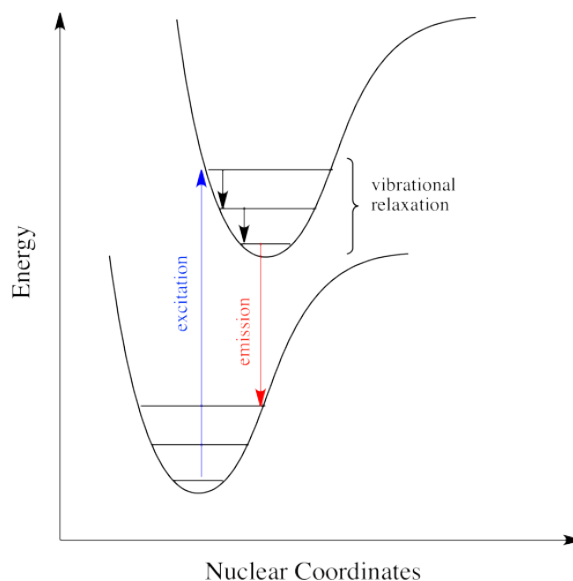


Figure 1.3: The Franck-Condon principle. Transitions of electrons to and from excited states in a molecule occur as if the nuclei were all stationary. [8]

The excitation of the molecule leads a change in the distribution of the electrons - they move further apart. As a result there is a transition to a higher vibrational state than previously. Relaxation rapidly takes place to the lowest vibrational state of the electronic excited state which causes a red shift in the fluorescence spectrum as there is less energy than it was excited with. This is known as Stokes' shift and should be noticed after a lifetime of a few nanoseconds in the case of larger molecules like chlorophyll. The energy lost in the vibrational transition may be received by the neighbouring proteins for reorganization, dissipated as thermal energy or transferred to another molecule in the sample.

It will also be constructive to mention Kasha's Rule which states that the same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength [9]. This will be seen clearly when comparing the two datasets in the results section, which were obtained using two different excitation wavelengths (Figures 2.1 and 2.2).

1.3.1 Factors influencing the absorption

Absorption of a photon will only occur if it has enough energy to raise the molecule to an excited state by a transition that is allowed. However, this is not all that determines whether or not a photon will be absorbed. The probability of absorption also depends on the relative position of the electric dipoles that exist in the molecules in relation to the external electromagnetic field which in this case is due to the laser passing through the sample. The dipole moment needs to be parallel to the electric field for maximum probability of absorption but absorption may occur as long as there is a component of the dipole in the direction of the electric field but at a reduced probability.

Spin also has a part to play in influencing the probability of absorption. Electrons all have a spin magnitude of a half which relates to the angular momentum of the electron and the orientation of the spin can either be parallel or antiparallel to the magnetic field. It is the total spin of the molecule that determines the overall spin state. This requires

adding the spin vectors of the unpaired electrons in the molecule. The magnitude of the total spin is given by S , from which we can find the spin multiplicity given by:

$$\text{Spin multiplicity} = 2S + 1$$

If the spin multiplicity equals zero then the molecule is said to be in a singlet state which occurs when the projection of the total spin vector onto the magnetic field vector is zero. As the name suggests, there is only one spin state describing a singlet state. A triplet state exists if the spin multiplicity equals one, which means it has 3 possible spins states that satisfy this spin multiplicity. The ground state of the molecule is a singlet state which means that all of the electrons in orbitals are paired with other opposite spin electrons. For excitation from ground state to a higher excited singlet state to occur, an electron just requires the right amount of energy to make the transition, conserving its spin orientation. As for excitation to a triplet state, not only does an electron require the correct energy to make the transition but it also has to change spin orientation. Changing between a triplet and singlet state, as is the case in intersystem crossing (ISC), requires a forbidden transition to be made and thus it greatly lowers the probability of photon absorption from the ground state (a singlet state) to a triplet state and causes ISC transitions to be less probable, thus lengthening the lifetime of these transitions.

Transitions are not only restricted to obeying spin selection rules but orbital symmetry also plays a role in whether or not a photon is absorbed. There are three main types of orbitals in molecular orbital theory: the σ orbital, the π orbital and the non-bonding orbital (n). The σ orbital is found by the combination of the s orbital wavefunctions of the atoms in the molecule. Likewise the π orbital is the orbital that results from mixing the wavefunction of the p orbitals in the constituent atoms of the molecule [7].

As the lower energy σ orbitals are already filled with electrons, π and n orbitals are of greatest interest in spectroscopy since electrons in these orbitals are free to be excited. The transition of an electron from one orbital to another of higher energy depends on the overlap of the wavefunctions of the electron in the initial state (Ψ) and the final state (Ψ^*). The greatest probability of a certain transition happening corresponds to the case where Ψ and Ψ^* overlap the most. Since π and π^* orbitals have the greatest correspondence, absorption of a photon resulting in $\pi \rightarrow \pi^*$ is allowed whereas the $n \rightarrow \pi^*$ transitions are symmetry forbidden due to negligible overlap of the wavefunctions of the initial and final states. In reality, however, due to the vibronic coupling that exists in the molecule, the $n \rightarrow \pi^*$ transitions have a small probability of occurring but the absorption occurs at a longer wavelength than the $\pi \rightarrow \pi^*$ transitions [7].

The intensity of the incident beam also contributes to the absorption in the sample. There being more photons to be absorbed by the sample in a high intensity beam thus more molecules are able to absorb the photons at any given time which results in stronger absorption.

1.4 Photosystem II

Photosystem II is a supercomplex found in plants consisting of many proteins with the twofold purpose of harvesting light and generating an electron and proton gradient,

through charge separation, in order to store energy. It needs to be an adaptable system due to the varying 'stress' conditions that it is exposed to nature. Stress conditions include exposure to intense sunlight on the plant, extreme heat or cold, the occurrence of a drought etc. For this reason it is essential for the plant to be able to adjust on a molecular level, as the process of photosynthesis depends highly on these conditions. The antenna system is responsible for this regulation to prevent possible damage from these stress conditions.

It consists of around 4 LHCII trimer complexes and other minor antennas and a reaction centre [10]. The LHCII complex is what is of interest to us and thus will be explored in more detail.

1.4.1 LHCII

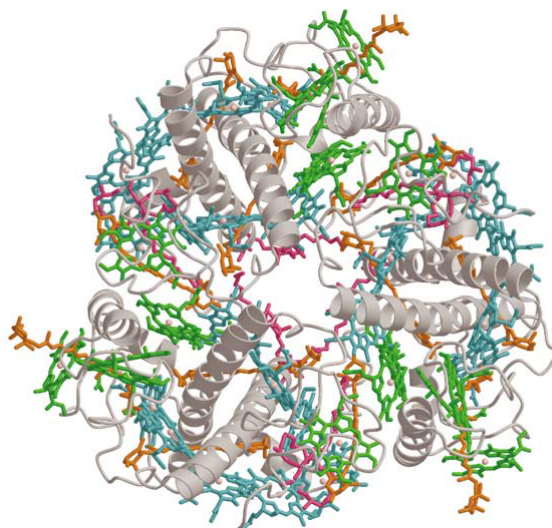


Figure 1.4: The structure of the LHCII trimer showing the carotenoids (orange), Chla (cyan) and Chlb (green). [11]

Of all the antenna proteins that are present in the photosynthetic systems in higher plants, the Light Harvesting Complex of photosystem II (LHCII) is the most plentiful [12]. It is a trimeric (consisting of 3 essentially identical subunits or proteins) pigment structure that is tightly packed and each monomer (subunit) in LHCII consists of, on average, 8 Chlorophyll a (Chla), 6 Chlorophyll b (Chlb) and 4 carotenoids : 2 Lutein, 1 Neoxanthin and 1 Violaxanthin, which are all light-harvesting pigments [13][11][14]. See Figure 1.4.

This antenna system is responsible for absorbing photons and ensuring that the energy from each is transported to the reaction centre with maximum efficiency, which is close to 100% ! Strong excitonic couplings exist between the pigments in LHCII. As the organizational structure of the LHCII complex can be subject to minor changes, the optical properties may change from one sample to another. With this flexibility in mind, the preferential pathways that the energy takes to the reaction centre are influenced by the location of the pigments with respect to one another - a carotenoid is more likely to transfer its energy to a chlorophyll that is a close neighbour. The overlap between

the emission spectrum of the excited pigment and the absorption spectrum of another pigment also impacts the probability of a transfer between the two occurring.

Chlorophylls

Chlorophyll a has 3 absorption bands, shown in Figure 1.4 the Soret band, absorbing in the wavelength region corresponding to blue - the higher energy transitions, with a maximum peak at 437nm. The next is known as the Q_x band, absorbing at a wavelength of around 620nm and finally the Q_y band absorbs in the red wavelength region with a peak at 675nm for the lower energy transitions [1]. The peak wavelengths of the absorption

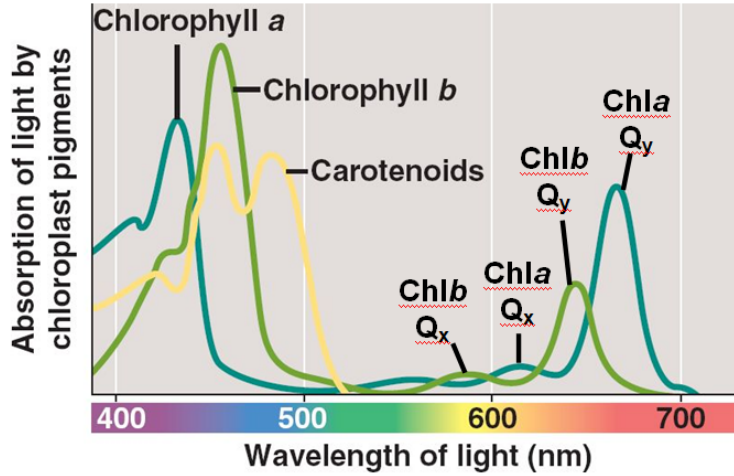


Figure 1.5: The absorption bands for Chla (turquoise), Chlb (green) and the carotenoids (yellow). [15]

bands, depend on what solvent that the chlorophylls are diluted in. Two Chla pigments in the reaction centre are responsible for the separation of charges that takes place in the conversion of light energy into chemical energy while the other Chla molecules in the photosystem harvest light..

Chlorophyll b is an accessory pigment meaning it is not directly involved in the conversion of light energy to chemical energy. It absorbs in a slightly different region as can be noted in Figure 1.5. The absorption bands are shifted closer together with the peak in the Soret band being present at around 470nm, the Q_x band absorbing at around 600nm and the Q_y peak at around 650nm [16]. Chlorophyll b transfers its electronic excitation energy on to the Chla's on a picosecond time scale. The differences in the absorption spectra of Chla and Chlb is attributed to the slight molecular difference between the two pigments. Chlorophyll b closely resembles Chla but it replaces the CH₃ found in the top right location of the Chla (in Figure 1.5) with a CHO group. This replacement changes the distribution of charges in the two molecules and thus Chla and Chlb have different dipole moments. The transition dipole moment of a molecule is a measure of the probability of a transition taking place and is given by:

$$\mu_{fi} = \int \psi_f^* \boldsymbol{\mu} \psi_i d\tau$$

where ψ_f and ψ_i are the wavefunctions for the initial and final states and $\boldsymbol{\mu}$ is the electric dipole for the charge distribution of the molecule. Molecules in the states Q_x and Q_y

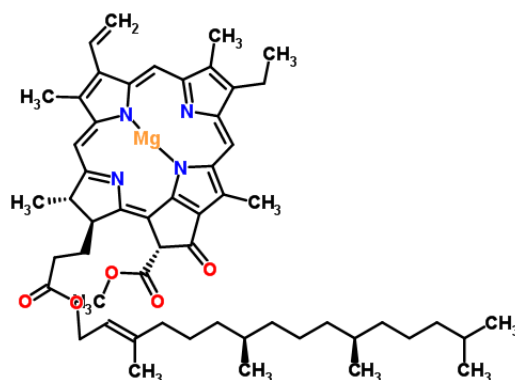


Figure 1.6: The molecular structure of Chla. [17]

have different charge distributions and thus their electric dipole moment is different. The Q_y state has a stronger dipole moment and hence the transition dipole moment is greater than that for Q_x making transitions to Q_y far more probable. This is reflected in the amplitudes of these peaks in the absorption spectrum.

Carotenoids

The carotenoids (Cars) are also accessory pigments in LHCII and they have a number of purposes in photosynthesis. They assist in absorption, as the carotenoids absorb light in the blue/green region, with a different range of wavelengths to the chlorophylls (refer to Figure 1.4). Note that there is little absorption by any of the pigments in LHCII in the wavelength range for green light, that is why leaves are the colour green because these wavelengths get reflected by or pass through the leaves. They are a vital part of transferring excitation energy to the reaction centre in the photosystem and the Cars are also integral in quenching chlorophyll triplet states (via van der Waals interactions) which would otherwise result in high energy singlet oxygen forming which is harmful to biological systems [18][12]. This excess energy as a result of quenching is dissipated as heat [19].

The low-lying excited states, S_1 and S_2 , are singlet states. Transitions from the ground state, S_0 , to S_1 are forbidden because they have the same inversion symmetry but internal conversion can take place between $S_2 \rightarrow S_1$. Excited Cars in the S_2 state have a fast transition to either higher Q_x or the lower Q_y energy level of the Chls (refer to Figure 1.9) and account for most of the energy transfer to the Chls [14]. This is primarily due to the dipole-dipole interactions between the S_2 state and the vibronic states of the Q band of the Chls [1].

The overlap between the fluorescence spectrum of the Cars S_2 state and the absorption spectrum for Q_y state of the Chls is fairly small which means it is more likely that the Q_x state of the Chls will be the acceptor of the excitation energy, since this peak is energetically closer to the blue-green Car region. The S_1 state of the Car also transitions to the Q band in the Chls but this is not the most preferential pathway for energy trans-

fer with $< 20\%$ of the excitation energy being transferred this way [1][14]. This is a fast transfer to the neighbouring Chls but less probable.

The Cars that are of importance in LHCII are Lutein, Neoxanthin and Violaxanthin. The structure of these molecules can be seen in Figure 1.6. The spectral properties of a

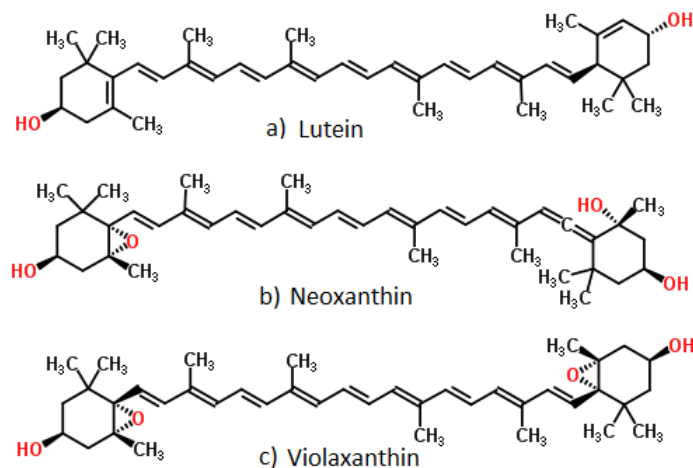


Figure 1.7: The molecular structure of the carotenoids. [20]

molecule change depending on the number of conjugated π bonds in the molecule (these are the chain of bonds in the middle region of the Cars in the figure). Lutein (Lut) principally absorbs at a wavelength of 494nm and it also serves to stabilize the LHCII complex [1][12]. It interacts exclusively with Chla, via van der Waals forces which leads to efficient light harvesting and photoprotection [11]. Two Lut are located in the central binding sites of the LHCII complex and 6 Chla's are situated closely around these [12]. Due to their close proximity, singlet energy transfers from Lutein to Chla have a high probability of occurring [11]. Neoxanthin (Neo) has an absorption peak around 486nm and transfers energy principally to Chlb. Finally, Violaxanthin (Vio) absorbs maximally at 510nm and transfers its S_2 state energy chiefly to Chla [1]. It is also important to note that there is an overlap between regions where the Cars absorb and the absorption of the Soret bands for the Chls.

Protein environment

Proteins are disordered and their ground state has a high degree of degeneracy. For this reason, there are a large number of substates that the proteins can possess. This directly effects the absorption properties of the pigments coupled to the proteins and thus there are slight variations in the absorption spectrum of pigments in a protein environment. The LHCII is an example of such a system where all of the transitions and energy transfers are in the context of a system of molecules coupled to proteins. The energy loss in the molecule through processes such as vibrational relaxation, can be used for to reorganise the protein environment and is termed the reorganisation energy, λ . As the molecule relaxes, its charge distribution changes and hence its dipole moment. In order to conform to this change, the neighbouring proteins undergo a reorientation using the reorganization energy to power these changes. This is due to the coupling between the states in the pigments and the vibrational modes of the nuclei in the protein.

1.5 Energy pathways in Photosynthesis

The term ‘lifetime’ in the context of this project and in the subject of absorption is the time between the absorption of a photon by a molecule and the emission of a photon at a later stage. This is the most probable amount of time that a molecule will take to emit a photon from a specific state.

There are a number of pathways that the energy can take in order to get to the reaction centre. These pathways need to be outlined in order to understand what takes place in LHCH. The main processes within a molecule are summarized in Figure 1.8

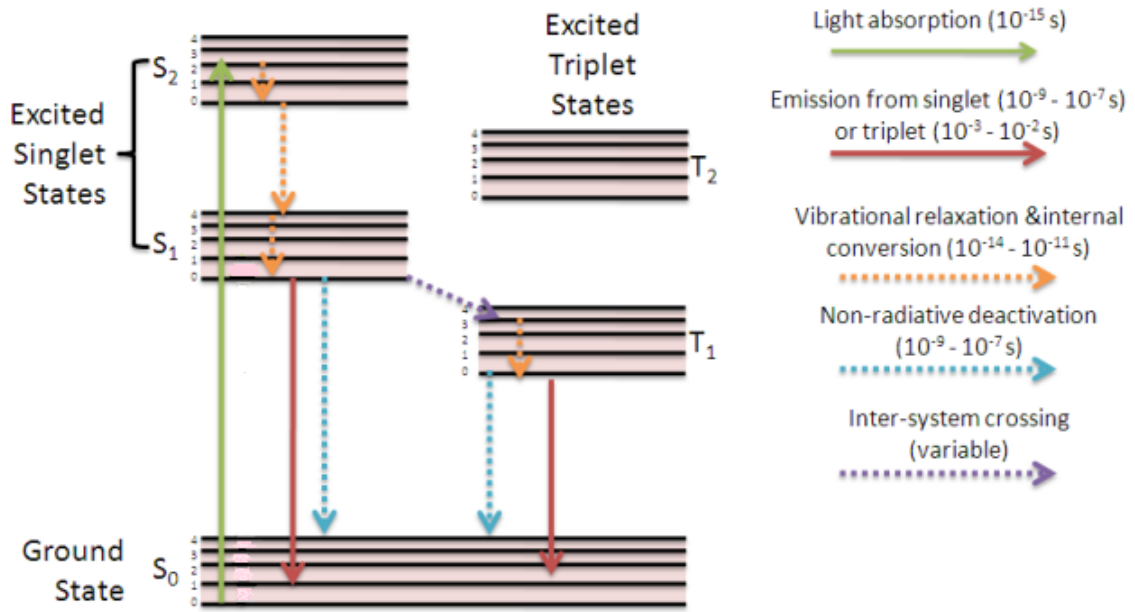


Figure 1.8: Energy transfer pathways that occur within a molecule. [21]

Upon excitation, the molecule is raised to the S_2 state or higher states since transitions $S_0 \rightarrow S_1$ are forbidden in carotenoids, due to the states having the same symmetry. A result of the Franck-Condon principle, the molecule in S_2 will have a non zero vibrational state and then undergoes vibrational relaxation. This generally takes place in the range of 100fs to a couple picoseconds.

In the S_2 state there are two possible pathways to take within the molecule itself to return to the ground state. Either this singlet state can decay to a triplet state in a process known as intersystem crossing (ISC) or it can decay to the lower singlet S_1 state in a process called internal conversion (IC). These are both radiationless transitions. Internal conversion can also take place to the ground state but this is a lot less probable so it has a much longer lifetime in the order of nanoseconds [22].

The excited states can also return to their ground state via radiative energy transitions whereby a photon is released in a process called fluorescence (when emitted from a singlet state), or phosphorescence (when the photon is emitted from a triplet state). The emitted photon has a frequency corresponding to the energy difference between the

current excited state and the ground state of the molecule.

Phosphorescence is less probable so it has a longer lifetime since it requires changing the state from that of a triplet to a singlet. On rare occasions a molecule in an excited triplet state can transition into an excited singlet state via ISC and only then undergo fluorescence to the ground state. In this case this is delayed fluorescence and this takes place much longer after absorption of the photon has occurred, even as long as milliseconds after absorption [22].

Lastly, energy equilibration is the process whereby a molecule reaches a state of lowest energy and for the complex system of LHCII under consideration, energy equilibration takes place through the convoluted interplay of pigment-pigment and pigment-protein interactions.

The matter of energy pathways becomes more complicated when the energy transfer between molecules is taken consideration. Figure 1.9 summarizes the entanglement of

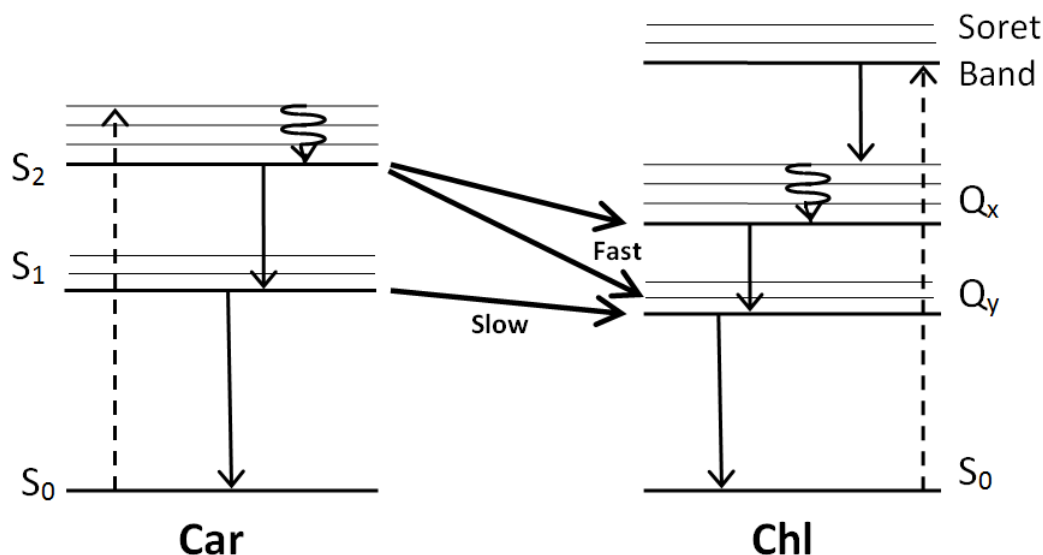


Figure 1.9: A simplified overview of the complex nature of the energy transfer pathways between the Car and Chl molecules

the different pathways that can be taken by the Cars and Chls to return to their ground state after excitation. The process of intersystem crossing is left out for the sake of the simplicity of the figure. These pathways are in competition and there tends to be a "bottleneck" effect at the Q_y energy level. The preferential transfer channel at a bottleneck state is that which has the highest probability for that transition.

1.6 Pump-probe spectroscopy

The ExciPro¹ femtosecond transient absorption system consists of a pump-probe optical unit and the software necessary for computing the difference absorption spectrum for a

¹Further specifications of the ExciPro can be found in the *Femtosecond pump-probe system, ExciPro User Manual* 2004-2007 CDP Corp.

sample. A detailed picture of the setup is shown in the Appendix 4.1 in Figure 4.2.

The Beer-Lambert law is required as a starting point to derive the formula necessary for the ExciPro to compute the difference absorption spectrum. The intensity of the incoming light is altered at the sample as some of the energy is absorbed when passing through the diluted LHCII sample. Thus the incoming intensity, I_{in} , is greater than the outgoing intensity, I_{out} . The relation between these two intensities is known as the transmittance given by:

$$T = \frac{I_{out}}{I_{in}} = 10^{(-\epsilon c l)} \quad (1.2)$$

Where c is the concentration of the sample and l is the path length that the light beam travels through the sample. Here ϵ is the extinction coefficient which is intrinsic to the substance and represents the probability of absorption occurring for that specific molecule. The larger ϵ is, the greater the absorption in that molecule. The extinction coefficient also increases with a greater number of conjugated carbon bonds there are in a molecule [22]. The transmittance can also be written in terms of power through the cross sectional area of the laser pulse passing through the sample: $T = \frac{P_{out}}{P_{in}}$.

The absorbance can be found by taking the logarithm of both sides of equation (1.2) yielding:

$$A = OD = \log\left(\frac{I_{in}}{I_{out}}\right) = \epsilon c l$$

$$OD = -\log\left(\frac{I_{out}}{I_{in}}\right) \quad (1.3)$$

which will be developed a little later into the form required for computing the difference absorption spectrum.

As the name suggests there are two laser pulses involved in pump-probe spectroscopy: the pump and the probe pulse. The pump pulse is responsible for exciting the sample of LHCII and the probe pulse, which is delayed in time, has the purpose of monitoring changes in absorption of photons by the sample and it spatially overlaps the pump pulse at the sample. The basic setup is seen in Figure 1.10 Time zero corresponds to the point in time where the pump and probe pulse overlap temporally, after this time the probe pulse is delayed and changes in the absorption can be investigated at some time later, after the initial excitation. The duration of the pump and probe pulse place a limit on the fastest energy transfers that we can observe, thus the shorter the pulses the faster the dynamics that can be observed. Although, in accordance with the Heisenberg's uncertainty principle, better time resolution diminishes the spectral resolution.

The probe pulse has to satisfy a number of conditions for this type of spectroscopy. The probe is a lower intensity pulse in order to avoid sample damage and allows to observe the changes in absorption due to photo-induced transitions in the sample. Apart from spatial overlap with the pump pulse, the diameter of the probe pulse must always be smaller than that of the pump to ensure only the molecules in the region of the initial

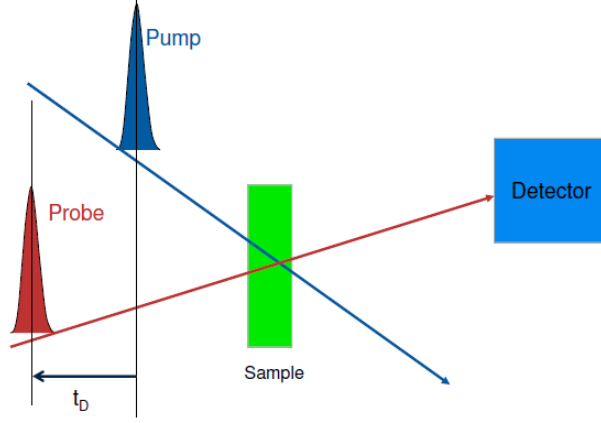


Figure 1.10: The pump and probe pulses as they overlap at the sample. The probe pulse excites the sample at time t_D after the pump and goes through to the detector. The pump probe is terminated after the sample [23].

absorption are probed for changes in absorption. The probe pulse must be able to probe a broader range of wavelengths, that is to say be spectrally broader than the pump pulse so that the absorption changes can be monitored over a larger range of wavelengths to better understand the energy transfers that take place in the sample after excitation. The probe pulse that was used for these experiments was a white light, generated from a sapphire crystal before the sample. In theory, the absorption changes across all the wavelengths of visible light could be observed however, due to other factors of the setup, the range of wavelengths that could be recorded for was restricted to 220nm per experimental run.

The pump pulse excites the Car pigments at a certain wavelength from their ground state, S_0 , to their second excited state, S_2 , and a photodiode detector system detects the changes in absorption in the visible light range based on measuring the intensity before and after the sample. The spectrum that we are concerned with is that of the change in absorption or also termed the change in optical density ΔOD . The change in absorption is given by:

$$\Delta OD = A_{pump-on} - A_{pump-off} \quad (1.4)$$

Where $A_{pump-on}$ is the absorption of the light in the sample when the pump pulse is responsible for the excitation and $A_{pump-off}$ is the absorption of incident photons in the sample when “not pumped”. With this in mind, ΔOD is negative when $A_{pump-on} < A_{pump-off}$ in a region, which corresponds to signal such as Ground State Bleach (GSB) or Stimulated Emission(SE). In the presence of the pump pulse, many molecules that absorb in the region of the pump pulse bandwidth are raised from their ground states to excited states, leaving fewer molecules in their ground state. When the probe pulse is incident on the sample, there are fewer molecules in their ground state to be excited and thus less absorption occurs - a decrease in the absorption between pumped and not pumped so the ΔOD will be negative. In the case of stimulated emission, the probe pulse stimulates an emission of a photon in the excited molecules and thus the absorption decreases (since a photon is emitted not absorbed).

A positive ΔOD occurs when the $A_{pump-on} > A_{pump-off}$ which corresponds to the case of excited state absorption, which means there is further absorption that takes place from

the excited states. As the electronic energy levels get closer to one another with higher excited states, the energy required to further excite a molecule is less. Thus the energy absorbed in the presence of the pump pulse is greater than absorbed when the probe excites molecules to higher states and thus is a positive signal.

1.7 Data Analysis Methods

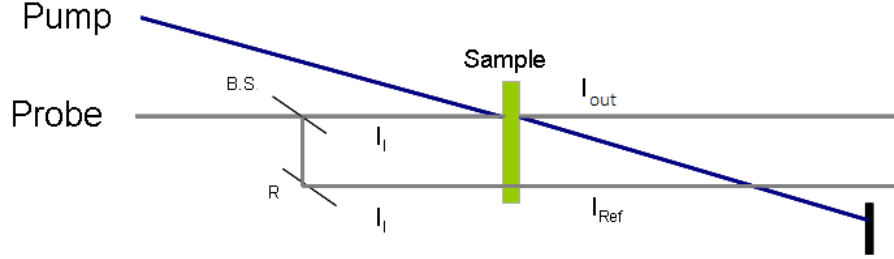


Figure 1.11: The setup used to determine the difference in the optical density. [23]

With reference to Figure 1.11 a reference beam, which passes through another area of the sample, is used to determine the input intensity, I_{ref} , and the output intensity, I_{out} , that is received by the photodiode array and the ExciPro program computes the change in absorption at each time delay according to the combination of equations (1.3) and (1.4):

$$\Delta OD(\lambda) = \log\left(\frac{I_{ref}}{I_{out}}\right)_{ex} - \log\left(\frac{I_{ref}}{I_{out}}\right) \quad (1.5)$$

where the subscript $_{ex}$ refers to the absorption when the sample is excited by the probe beam (i.e. “pumped”). With a little rearranging, equation (1.5) can be written as:

$$\Delta OD(\lambda) = -\log\left(\frac{\left(\frac{I_{out}}{I_{ref}}\right)_{ex}}{\left(\frac{I_{out}}{I_{ref}}\right)}\right)$$

The denominator is the ratio of intensities when the pump beam is temporarily blocked and therefore the sample is “not pumped”. This is achieved by a device called the chopper which is synchronised in the ExciPro setup. As the time delay varies the ΔOD evolves and a plot is recorded containing the optical density information for every wavelength in the recording width of the window as a function of time.

Glortaran

Glortaran² is a very powerful and well developed program specifically designed to analyse and model femtosecond spectroscopy data. It uses spectral methods to model the spectrum, $\Psi(\lambda)$, given by:

$$\Psi(\lambda) = \sum_{l=1}^{ncomp} c_l \epsilon_l(\lambda) + v(\lambda)$$

²For further documentation see (1) <http://glortaran.org/> (2) J. J. Snellenburg, S. P. Liptonok, R. Seger, K. M. Mullen, I. H. M. Stokkum, Glortaran: A Java-Based Graphical User Interface for the R Package TIMP, *Journal of Statistical Software*, 49:1-22, 2012

Where c_l is the unknown contribution of the l th component that the program determines, ϵ_l is the known spectrum of the l th component from the data and $v(\lambda)$ is the additive, normally distributed noise that the system may encounter.

For the purposes of this project, only global analyses were conducted on the data as a target analysis requires the setup to have a much better signal to noise ratio. A global analysis models the data assuming sequential decay, meaning that each kinetic state of the system as a whole decays successively into one another. This results in a few simple models with estimated values of the lifetime for each component. The implication of this is that the kinetic models that are obtained are mixed states (as a result of multiple decay pathways combined). A target analysis, which gives the combined results of a parallel and sequential model, produces all the independent kinetic states involved in the decay. The spectrum that we are concerned with is the Evolution Associated Spectra (EAS), which represents how each components spectra evolves in time as a result of the decay or rise in energy of the other kinetic states.

Applying this knowledge to the datasets at hand, there are 4 components that were able to be resolved for each dataset. This means that each term in the above series represents one of the 4 spectra in each dataset and each of these so called components sequentially decay into the next, $1 \rightarrow 2 \rightarrow 3 \rightarrow 4$, each with their own lifetime τ_l . Figure 1.12 shows this.

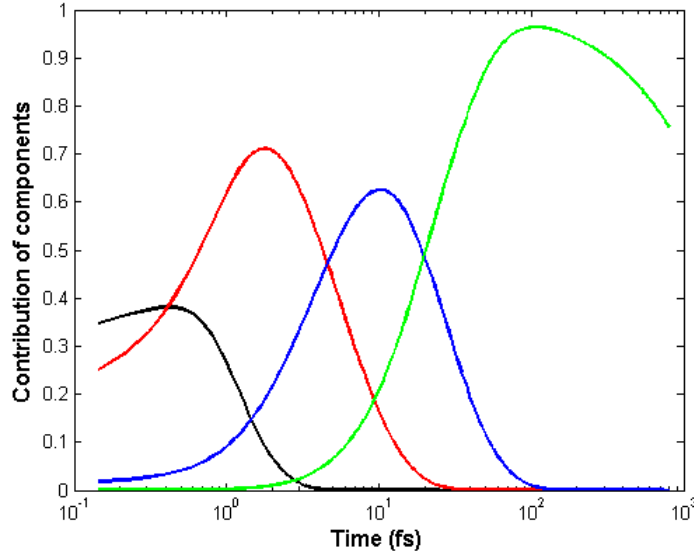


Figure 1.12: The individual components resolved by the Glotaran sequentially decay into one another over time.

1.8 Understanding the Data

With the above in mind and a better understanding of what GSB, SE and ESA are, we can turn our attention to understanding how this relates to the results that will be seen in this project. The following section is the framework that will be built upon in the Results and Discussion section.

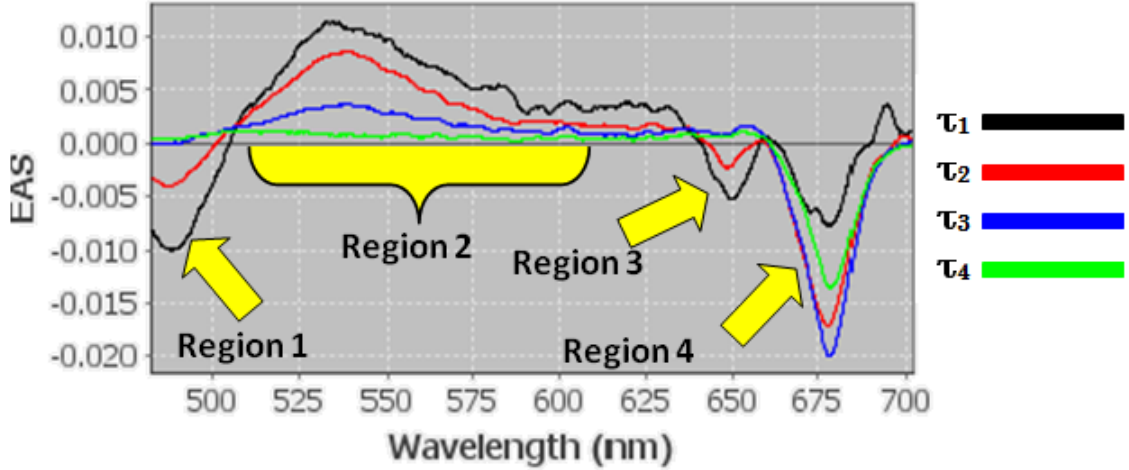


Figure 1.13: Shows one of the datasets that will be encountered in the results section. There are 4 regions that will be considered separately.

With reference to Figure 1.13, note that the vertical axis is the EAS and thus looking at how the kinetic states (the 4 components mentioned earlier) evolve sequentially from one to the next. Each state has its own lifetime given by τ_i . The black spectrum is the unrelaxed excited state, the red spectrum is the fast relaxed excited state, the blue spectrum refers to the slow relaxed excited state and finally the green spectrum represents the final component or time constant that Glotaran resolves.

At time zero the sample absorbs the incident light from the pump and the probe. It is at this instant that there is initially no change in absorption. What occurs after this is reflected in the EAS, the spectrum showing the evolutionary change in absorption. The wavelengths in Region 1 are where the initial absorption takes place as the excitation wavelengths for both of the datasets were in this region (with peaks at 494nm and 510nm). Thus this signal corresponds to the case of GSB where both the carotenoids and the high energy Soret band of the chlorophylls are excited. As the delay between the pump pulse and the probe pulse increases, the change in absorption becomes less negative as the populated excited states begin to decay via the various energy pathways resulting in more molecules being in their ground state to absorb the energy from the probe beam. As $\text{time} \rightarrow \infty, \Delta OD \rightarrow 0$ absorption. The carotenoids transfer energy on to Chls, causing excitation of the Q_y band of Chlb and Chla found in Region 3 and 4 respectively.

Region 2 indicates ESA by the positive signal at higher wavelengths. The energy required to raise a molecule to a higher excited state is less than that to excite it to the current state so hence this occurring in the wavelength region of lower energy. ESA decreases the more the probe pulse is delayed since the pump induced excited states decay leaving more in the ground state and less excited states for further excitation by the probe beam.

Chlorophyll b absorption occurs in Region 3. This negative signal is due to GSB as Chlb is excited to higher energy levels.

Finally, Region 4 shows the signal due to the absorption by Chla. Here GSB should be detected as the Chla absorbs initially but now as the delay between the pump and probe pulse increases, instead of tending to no change in absorption at longer time delays, we see that the change in absorption remains negative indicating a gradual transition from GSB to the dominant decay pathway of SE. This is further confirmed by the Stokes' shift that can be observed in the blue and green spectra. This being said, one must keep in mind though that SE is not the only decay channel at this point but, ISC and IC are also still feasible decay pathways.

1.9 Materials and Methods

1.9.1 Experimental Setup

The overall setup of the experiment can be found in the appendix. The coherent, near infrared beam produced by the Ti:sapphire femtosecond laser unit has a central wavelength of 775nm and bandwidth of 8nm. The pulse duration as measured by the autocorrelator was 150fs with a repetition rate of 1kHz. This restricts the fastest energy transfer that can be observed to take place in the sample to a value > 150 fs. The laser beam initially encounters a beam splitter where 90% of the beam becomes the pump beam and the other 10% becomes the probe beam. The pump beam is reflected to the Noncollinear Optical Parametric Amplification(NOPA)³ optical system, the characterization of which is beyond the scope of this project system. The end product of the NOPA though, is the pump beam with a different wavelength. Changing the wavelength of the light directly impacts which pigments are excited more preferentially since, as mentioned earlier, the absorption spectrum of each type of pigment is different. After this the pump beam is directed by a series of mirrors and optical lenses to the synchronised chopper, which controls when the sample is pumped and not pumped for recording the spectra. The pump pulse then enters the ExciPro setup where it is then directed to the sample to excite the LHCII complex and is terminated after this. The probe pulse must traverse a path length equal to that travelled by the pump pulse in order to arrive at the sample at the same time, so a series of mirrors are used to accomplish this until it reaches the delay line in the ExciPro. The adjustable optical delay line is what adds the delay in real time between the pump and probe pulse and consists of a retro-reflector attached to a computer automated delay stage. The smallest distance that the optical delay line can move is 2.1×10^{-7} m, corresponding to 0.78125fs. Each increment in distance introduces an additional delay depending on where the reflector is on the delay line which has the ability to range over 2ns delay in total. At each increment 5 readings were taken which ExciPro used to average over for better signal-to-noise ratio. From the optical delay line the probe beam is then reflected to the 2.15mm thick sapphire plate which generates a white light continuum before passing through the sample to a photodiode array.

1.9.2 Sample Preparation

The LHCII complexes had already been extracted and isolated from spinach leaves for the first dataset and the second data set was measured for isolated LHCII from Arabidopsis

³further characterisation of the NOPA can be found in the *NOPA, fundamentals and instructions*, ag-Riedle, Uni München 04/05.

thaliana and were obtained from a lab in Amsterdam. To prepare the sample for use, it had to be diluted in HEPES, a buffer which fixes the pH of the solution and thus sets the concentration of the Hydrogen atoms in the solution in order to best mimic the natural conditions of the photosystem, where the pH is close to 8. In the laser lab the main lights had to be switched off for the remainder of the experiment as the pigments in the LHCII sample are sensitive to light from any source so as not to damage the sample or cause excitation from a source other than that from the laser. The dark working conditions are also to prevent background light noise from being received by the detector. Working in low light, the $25\mu\text{l}$ sample was mixed together with $140\mu\text{l}$ of buffer. It was placed in a circular glass cell of width 0.5mm which was then placed in a rotator in the line of the pump and probe laser pulses which had been adjusted to overlap spatially on the sample. The circular glass cell had to be rotated so that the a new set of pigments in the sample could be excited every time it is pumped. This serves two purposes, the first being to reduce the number of potential long-lived states (triplet states of the Cars) which have a lifetime of a few microseconds [12]. Secondly, to reduce the likelihood of degrading the sample by exposure to too much light. The power of the pump pulse was measured just before it was incident on the sample and just after exiting the sample. This measurement took place at the beginning of the experiment and at the end using a power meter, consisting of a photodiode connected to a controller. This was to monitor whether the power was too large so as to guarantee the sample was not damaged and that the ΔOD was not saturating, as well as to prevent the occurrence of multi-phonon absorption which introduces further complexities in the modelling of the data. Finally, the absorption spectrum, OD, was measured via the spectrometer of the Ocean Optics software to ensure that the sample hadn't degraded in the time taken to run the experiment. It is important to take this measurement since biological molecules are unstable when isolated from their natural environments. A shift in wavelength of the absorption bands in the Car region or the Q_y band of the Chls indicates possible sample degradation. Another indication would be if the ratio between the Chlb and Chla Q_y bands is different to what it was before the experiment.

Chapter 2

Results and discussion

As this is global analysis, the figure represents mixed states of the energy transfer. This type of analysis scheme does not fit the specific/individual energy transfer pathways. The ultrafast energy dynamics ($<150\text{fs}$) could not be resolved due to the width of the probe pulse being 150fs but the other ultrafast (sub-picosecond) dynamics could be determined. In Glotaran, 15 iterations were looped through in which the program could converge to a solution of a accurate fit to the data points. The goal is to have a fast convergence so as to ensure an optimal fit. After the 5th iteration Glotaran had converged to a solution.

2.1 Excitation wavelength: 494nm

The first component that the model fits occurs at 670fs after the initial absorption of the photons.

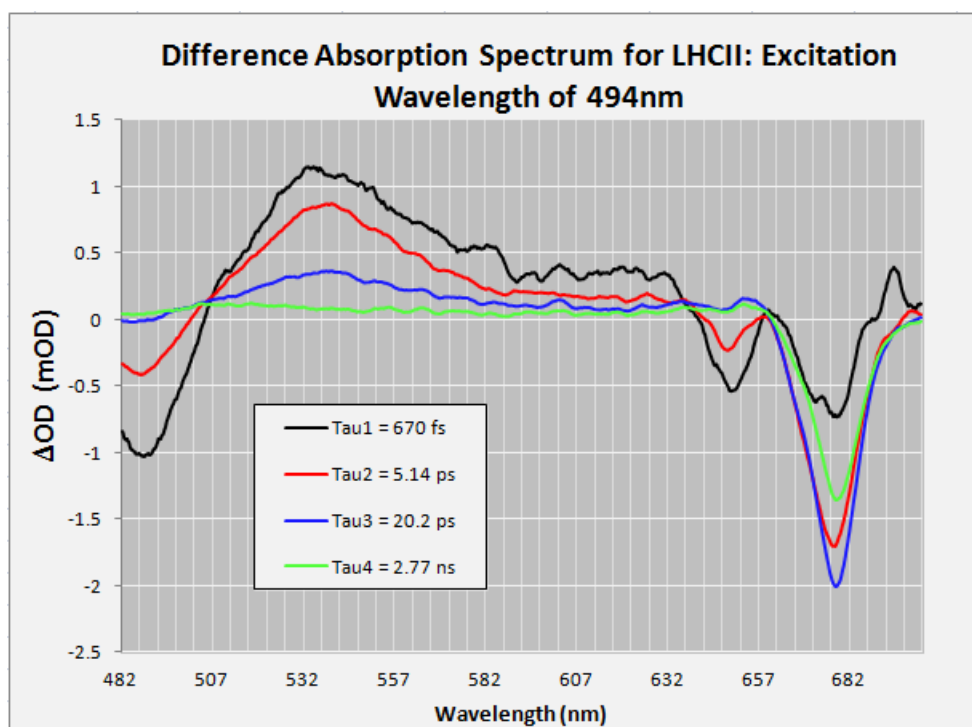


Figure 2.1: The difference absorption spectrum for LHCII extracted from spinach and excited at a wavelength of 494nm





Lifetime (ps)			
	τ_1	0.67 ± 0.33	
	τ_2	5.135 ± 0.035	
	τ_3	20.236 ± 0.089	
	τ_4	2765.6 ± 9.5	

Table 2.1: The lifetimes of the 4 components with uncertainties

The results for the parameters are shown in Figure 2.1 as well as Table 2.1. Analysis via a visual inspection can be determined first. This gives a rough outline of what is happening onto which a more solid analysis can be undertaken later. The pump pulse has a broad bandwidth so at an excitation wavelength of 494nm mainly the carotenoid region is excited with a slightly higher probability of the Lutein pigment being excited. From the carotenoid region the energy is transferred to the Chls on various time scales depending on which pathways the energy takes. From Figure 2.1 there is initially ground state bleach (GSB) of the carotenoids which reaches its maximum in the first time series (Black spectrum). As the window for this experiment was not large enough we are unable to see the rest of the GSB peak in the carotenoid region. This could have been solved by measuring 2 wavelength windows but due to the time limitations of the project this was unable to be done as fitting this data would have required a fair amount of additional work.

It can be seen in the spectrum for the first lifetime there is a positive signal ranging over 505-635nm which indicates ESA, which is also a maximum in the first time series. Further excitation occurs due to the probe beam in the carotenoids since at around 670fs delay there is still a densely populated number of excited Cars. There is a rise in the GS bleach in the Q_y region of the Chls at 650nm and 680nm, at this point in time it is not yet due to energy transfer from the Cars as their excited states have not begun to depopulate yet. Rather this rise is due to internal conversion from excited state in the Soret band of Chlb and Chla to the corresponding Q_y . The excited Soret band is a result of the overlap of the absorption spectrum of the Soret band and the region that the pump pulse excites [1].

Looking at the second lifetime (red spectrum) of 5.14ps, which is a result of the first spectrum decaying to this component, it can be seen that the absorption peak for Chlb has decreased by just over 50% while the peak for Chla has close to doubled in size. This is caused by excitation energy being received by the Chla pigments from both Chlb and Car pigments. The initial GS bleach of the Cars has now decreased by 60% due to the transfers taking place to the Chls. There are now fewer excited Cars so the absorption due the probe beam increases resulting in ΔOD becoming less negative. As there are less excited Cars, the amount of excitation to higher states due to the probe pulse decreases leading to the ESA region decreasing by around 20%. It can be observed that there is a transfer from Chlb to Chla that takes place around this time as the Chlb GSB decreases and Chla GSB increases which is in agreement with literature [1].

The Q_y band of Chla reaches a maximum peak at the third lifetime (20.2ps) which leads to the depletion of all the Chlb excited states back to the ground state as excited energy

transfer takes place from Chlb to Chla. There is no longer any difference in absorption occurring in the Car region and the ESA has been reduced to 30% of its original value, with the remainder of the energy to be transferred onto the Q_y state of Chlas.

The decay spectrum of the final lifetime shows that all the remaining energy of the system has been transferred to Chla. The longer lasting transfer due to fluorescence leads to a minimum transfer lifetime of 2.77ns. The delay line can only span over 2ns so the absorption changes occurring after this cannot be determined. The value obtained by the Glotaran for this lifetime is thus a lower limit of the possible lifetime of this final component since according to literature fluorescence should occur at around 4ns [24]. The peak of Chla has been reduced by approximately 35% as the Chla excited states begin to depopulate via the dominant decay channel, SE, as well as by ISC and IC. There is minimal ESA since there remains a miniscule number of excited Car states. The Stokes' shift can also be observed from the spectrum in the red shift in the peaks at around 675nm, as the Chla undergoes vibrational relaxation through energy used for protein reorganization and hence emits a photon of lower energy than the one that was absorbed.

In the context of a plant, from the Chla's, the energy is transferred on to the reaction centre where charge separation takes place which is not under consideration here. For this experiment the LHCII complexes are isolated - there is no reaction centre to transfer the energy to so in this case the remainder of the excited Chla's decay predominantly through SE and results in changes in the protein environment around it.

2.2 Excitation wavelength: 520nm

This dataset (shown in Figure 2.2 and Table 2.2) was measured across two windows that had to be glued together for Glotaran to model 1 data set. Where the gluing of the two data sets occurs there is a loss of information that the program was unable to accurately fit thus there is the flat portion that between 595nm and 628nm where the solution that was assumed for this region was that of no change in the absorption. This also introduced further uncertainty into the results as can be seen by comparing the uncertainties in the Tables 2.1 and 2.2. It can be noted that there is a difference in the ESA region of the two data sets. This is due to less of the carotenoids region (489-510nm) being excited at this excitation wavelength (520nm) and thus the width of the ESA region would decrease as well as the amplitude of the ESA peak [1]. Kasha's Rule, considered earlier, comes into play here where changing the excitation wavelength does not change the wavelength range of absorption by the Cars and Soret bands. The window is 200nm wide and the truncation of the activity at the 500nm wavelength results in more than half of the excitation peak is cut off. This does not have a huge impact our understanding of the dynamics as the behaviour in this region will closely resemble that of the previous data set. The difference in the other spectral regions will be of greater interest to us.

There is a strong ESA and small bleaching of Chlb that occurs in the first time series around 714fs. As the excitation wavelength was further from the Neoxanthin region of absorption it may explain why the Chlb peak isnt as prominent as in the previous data set. Another reason for a smaller bleaching peak is due to less excitation of the

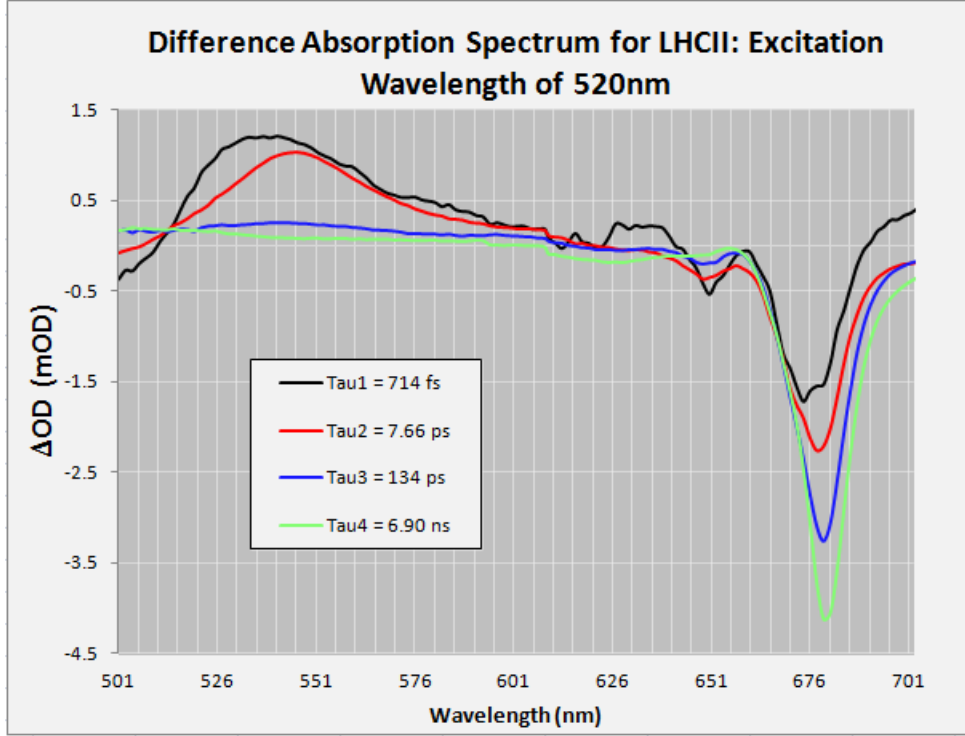


Figure 2.2: The difference absorption spectrum for LHCII extracted from *Arabidopsis thaliana* and excited at a wavelength of 520nm





Lifetime (ps)			
	τ_1	0.71 ± 0.29	
	τ_2	7.66 ± 0.19	
	τ_3	133.5 ± 5.5	
	τ_4	6897 ± 160	

Table 2.2: The lifetimes of the 4 components with uncertainties

Chlb Soret band as there is less overlap between the excitation wavelength range and that of the Soret band. Chla is the dominant recipient of the excitation energy due to the wavelength that is excited being closer to the absorption spectrum of the Vio and Lut carotenoids, which transfer almost exclusively to Chla. According to literature about 80% of the Lutein pigments would have transferred the energy absorbed onto the Chla's [14].

In the red spectrum, the second fastest mixed state has a lifetime of 7.66ps and the ESA has been reduced by approximately 10%, less than for the corresponding spectrum in data set 1 since there are fewer excited Cars. This peak should be closer to 540nm or 535nm but this skewing of this spectrum may be due to the gluing of the two data windows together that generated additional noise that influenced the fit of the spectral solution. There is a small reduction of about 10% in the region of Chlb which is again, less of a change than the previous data set since Chla is the preferential recipient of the excitation energy from the carotenoids. Thus the Chla GSB continues to increase. According to literature at around 1ps the Car S_1 states transfer energy over to the Q_y

states in the Chlorophylls so it is expected that there is a more notable increase in the absorption of Chla around this time but this is not well represented in this data set and only significant in the first data set [1].

By 134ps about 75% of the original ESA energy has been transferred and what is remaining decays much slower, possibly due to the long lived decay from S_1 to S_0 as this is a forbidden transition. The first data set shows much more pronounced ESA dynamics as a larger number of the carotenoids were being excited because of the chosen excitation wavelength. With the 3rd spectrum representing a lifetime 7 times that of the corresponding spectrum in the first data set, it is not unreasonable to see the dynamics of data set 1 tending towards the blue spectrum in the second data set.

In the final spectra there is another difference between the data sets. The green curve in data set 2 is larger than the previous spectrum which is mainly due to SE in addition to some GSB in the Chla region. The delay line for this experiment had a longer coverage than the one used in the first experiment but considering that SE should occur around 3 - 4 ns, the result of 6.90ns seems a little high. The difference in the final lifetimes may be due to the stability of the setup or it may indicate that the first data set was measured using a sample that had been degraded, perhaps as a result of the age of the sample. It could also be due to the fact that the LHCII was isolated from two different plant leaves or the differences in the solvent that the LHCII was diluted in (different concentration and possibly different constituents which also may explain why the fluorescence lifetime is longer).

Chapter 3

Conclusion

The global analysis of the energy dynamics in LHCII reveals just a part of the complex nature of the transfer of energy in the photosystem. There are many competing pathways that the excitation energy can take between Cars and Chls as well as between Chlb and Chla. These transfers take a place on timescales ranging from $< 100\text{fs}$ to a couple of nanoseconds and there are some decay channels that are more preferential at different times in this range. Not only do the pigments influence the absorption and interact with each other but the protein environment also play a part in how the energy dissipates in LHCII since there are strong couplings between the excited states of the pigments and vibrational states of the nuclei in the neighbouring proteins. The two datasets show good agreement with literature and they offer interesting insights into how changing the peak excitation wavelength affects the absorption and transfer of energy in LHCII.

It was found that, in the first resolvable lifetime, the ground state bleac that occurred at the Q_y state of Chla and Chlb on a sub-picosecond scale (670fs and 710fs for the first and second data set respectively) was due to internal conversion from their corresponding Soret band. The energy transferred from both Chlb and the Cars to Chla occurred within a few picoseconds (5.135ps and 7.66ps respectively) and after a couple of nanoseconds the depletion of excited states in the Cars and Chlb was evident having transferred their excitation energy to Chla. At this final lifetime fluorescence of Chla was observed in both datasets between 2.7656ns and 6.897ns .

As mentioned in earlier, one of the aims of this project was to get to grips with some of the concepts and ideas in the field of Biophysics and this has been achieved with the realization that what has been learned through this project is just scratching the surface of what actually occurs in PSII and in the interactions between pigments and proteins. There are a vast number of models (such as Redfield theory and the Generalized Förster theory) that, due to time constraints, I was unable explore and many other details that had to be left out of this report. All in all, Biophysics certainly is a fascinating field with the immensely challenging task of combining what is known in the biological realm with the field of physics, aiming to understand a very complex system one subsystem at a time.

Acknowledgements

I would like to thank my supervisor, Dr. Tjaart Krüger at the University of Pretoria, for the role that he played in this project as supervisor and mentor as well as for making the arrangements for me to conduct the necessary experiments at the CSIR National Laser Centre. Special thanks to Dr. Saturnin Ombinda Lemboumba for his generous gift of time and great assistance in the laboratory at the National Laser Centre. Mention should also be made of the Biophysics Group at the University of Pretoria with special thanks to Alexander Paradzah and Joshua Botha for the contributions that they made. I wish to convey my gratitude to Henny van Room from the VU University Amsterdam for the LHCI isolation from the spinach leaves and Pengqi Xu, from the same research group, for the isolation from Arabidopsis Thailana. I also wish to thank Dr. Williams for taking on the role of co-supervisor and for advising me on how to present this field to the Physics department at Rhodes University in an appropriate way.

Chapter 4

Appendix

4.1 Additional Figures

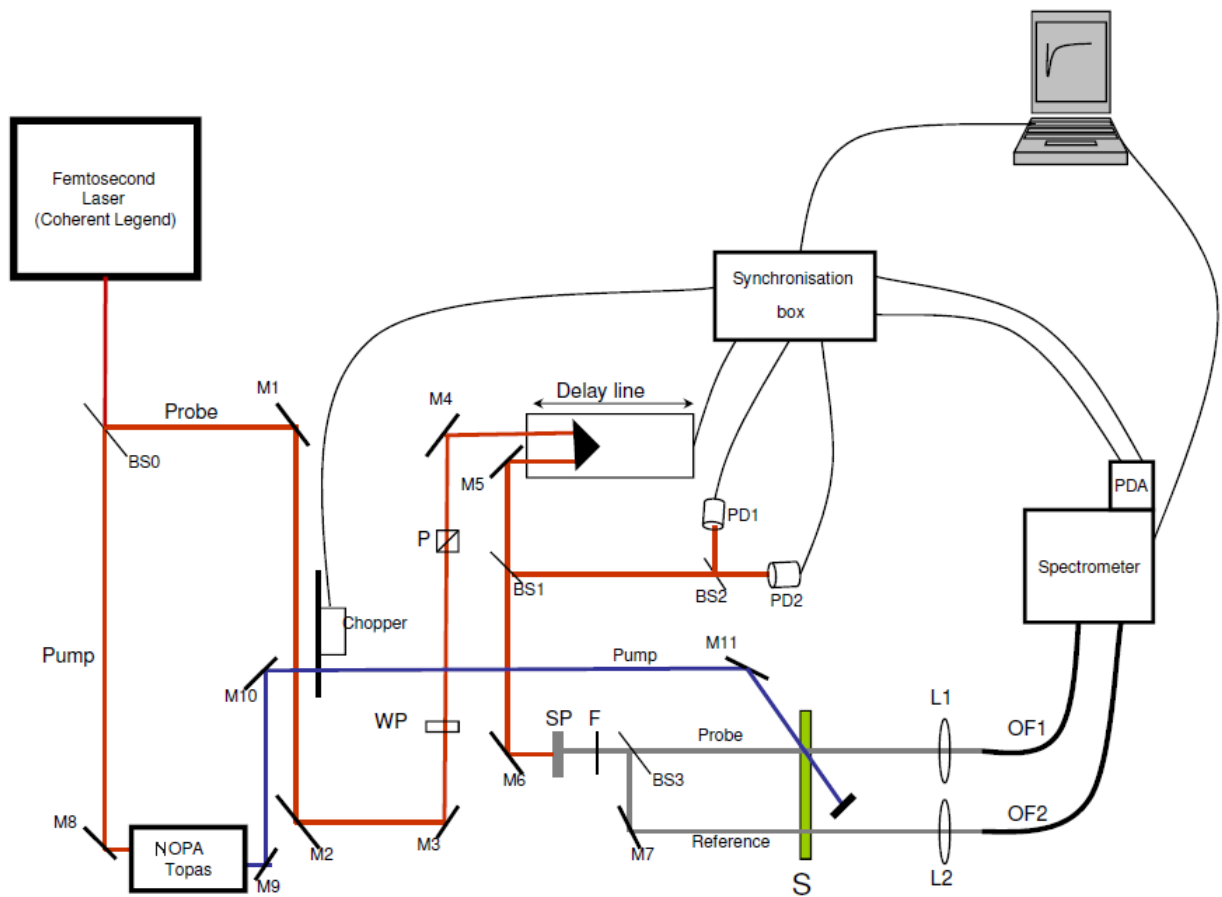


Figure 4.1: The path of the pump and probe pulse as they traverse a different path to the sample and photodiode array. The pump beam goes to the NOPA optics unit while the probe is reflected by a series of mirrors (M1-M4) until it reaches the delay line. Both the beams meet at the sample (S). [23]

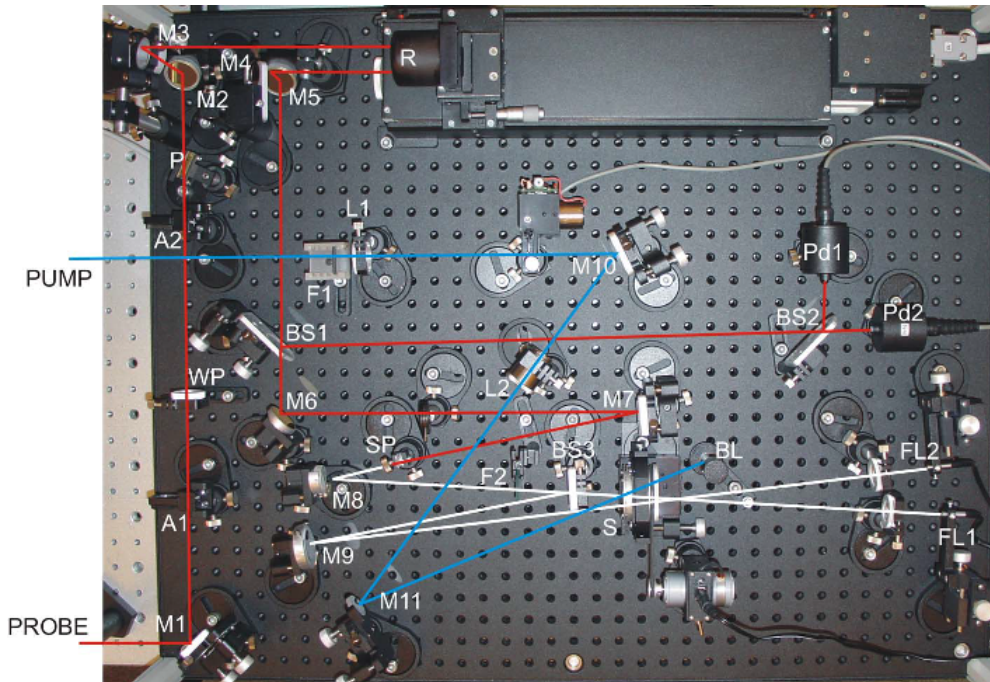


Figure 4.2: The path taken by the pump and probe pulse in the ExciPro setup. The delay line with the reflector plate (R) is situated at the top of the figure and the sample is placed at S. The sapphire plate (SP) is where the probe pulse becomes a white light continuum. [25]

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