Biophysical analysis of PS II – LHCII supercomplex

Understanding the LHCII phosphorylation-dependent dynamics along the thylakoid membrane

André Graça
This thesis is dedicated to my parents
Abstract

Plants have a degree of flexibility for many processes occurring at the chloroplast thylakoid membrane level which are essential for plant fitness and survival under changes in light quality and intensity. Phosphorylation of light-harvesting antenna complex II (LHCII) is known to induce such kind of processes, changing protein trafficking along the thylakoid membrane (state transitions), with the aim to better cope with light harvesting under new environmental conditions. Not many details are known yet about this dynamic that causes light energy redistribution between photosystems. By means of fluorescence correlation spectroscopy (FCS), I tried to determine the LHCII phosphorylation-dependent protein diffusion in thylakoid stroma membranes isolated from *Arabidopsis thaliana*, a higher plant. To address such possible differences, state transitions were induced for wild type plants and LHCII phosphorylation-deficient mutant STN7 was used as control. Unfortunately, such study presented many difficulties which make me state here that no conclusive answer to this question was found.

Additionally, in this study, STN8 mutant, known to lack the kinase responsible to phosphorylate photosystem II core proteins, was investigated along with wild type and STN7 strains. A successful characterization of the used biological objects was achieved by pulse modulated amplitude (PAM) and oxygen evolution, each of the results supporting each other. Also, novel fluorescence lifetime imaging results are reported for intact chloroplasts of the three analysed strains. My observation, in agreement with the known quantitative distribution of proteins on thylakoid membrane, suggests that the LHCII is the most abundant protein present on the membrane and its phosphorylation induces changes in protein conformation with the ability to extend the lifetimes of the fluorophores involved in light harvesting and energy transfer. When compared with the wild type, amplitude averaged lifetimes of the fluorophores are shown to be extended, nearly two times for STN7 mutant and an increase of 38% for STN8 mutant.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>b6f</td>
<td>Cytochrome b6f complex, part of the thylakoid electron transport chain</td>
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<tr>
<td>BBY</td>
<td>A method to isolate thylakoid grana membrane fractions developed by Berthold, Babcock, and Yocum.</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CBP</td>
<td>Chlorophyll Binding Protein</td>
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<tr>
<td>CCCP</td>
<td>Carbonylcyanide m-chlorophenylhydrazone</td>
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<tr>
<td>Chl</td>
<td>Chlorophyll</td>
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<tr>
<td>D</td>
<td>Diffusion Coefficient</td>
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<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethyl urea</td>
</tr>
<tr>
<td>DGDG</td>
<td>Digalactosyldiacyl-glycerol</td>
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<td>FAF</td>
<td>Fluorescence autocorrelation function</td>
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<td>FCS</td>
<td>Fluorescence Correlation Spectroscopy</td>
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<tr>
<td>FWHM</td>
<td>Full Width at Half Maximum</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence Lifetime Imaging</td>
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<tr>
<td>GMO</td>
<td>Genetic Modified Organism(s)</td>
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<tr>
<td>GUV</td>
<td>Giant Unilamellar Vesicle</td>
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<tr>
<td>LHCII</td>
<td>Light Harvesting Complex II, antenna</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyldiacyl-glycerol</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige &amp; Soog (Media plates)</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>PAM</td>
<td>Pulse Amplitude Modulated</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PPBQ</td>
<td>2-phenyl-p-benzoquinone</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
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<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
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<tr>
<td>S1</td>
<td>State 1</td>
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<td>S2</td>
<td>State 2</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis</td>
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<tr>
<td>SMD</td>
<td>Single Molecule Detection</td>
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<tr>
<td>SQDG</td>
<td>Sulfoquinovosyldiacyl-glycerol</td>
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<tr>
<td>TCSPC</td>
<td>Time Correlated Single Photon Counting</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
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Author contribution
The author states that most of the work presented in this document was performed by himself with the exception of the GUV production for FCS measurements. The author is profoundly thankful to Johannes Sjöholm, close collaborator from Jerker Widengren’s laboratory (KTH, Stockholm), for the production of GUVs.
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1. Introduction

The marvellous natural diversity was initiated by the first photosynthetic organisms more than 3.2 billion years ago [1]. But was the first photosynthetic organism the principal authority that shaped life as we know it today, or was the main role played by light itself?

On a macro-scale, visible light has been almost a constant throughout the 13.8 billion years of Universe’s existence. When the first photons were formed, between 240 000 and 300 000 years after the Big Bang, photons allowed the Cosmos to shine and fill up with amazing colours that we are capable to see today with our own eyes. Even restricting ourselves to the visible region of the electromagnetic spectra, we see light as a complex physical phenomenon which can be resumed to the pure result of excitation and relaxation of electrons. When electrons in different orbitals of atoms receive energy, if enough, that energy allows the electrons to move away from the atomic nucleus, to orbitals of higher energy. As everything in life requires stability, sooner or later the electron undergo relaxation processes, coming back to a state of lower energy by emitting energy in the form of a photon. Depending on the energetic states and the energy involved in this processes, energy might be radiative reemitted in the form of fluorescence.

Since the discovery of fluorescence, in the middle of the 19th century, the understanding of this phenomenon has extended the horizons of chemistry and biology, enlivening the field of photochemistry. There are countless spectroscopic techniques based in fluorescence and several requiring confocal microscopes – whose origin dates to 1955 – which allowed us to reach the detection limit of single molecule/particle, a bit later in the 80’s.

In this work, the involvement of light – in the form of fluorescence – in photosynthetic processes, is used and discussed. The following concepts will allow you to gather a better understanding of this thesis.

1.1. Oxygenic Photosynthesis

Photosynthesis is the origin to all oxygenic life, as we know it today. It occurs in plants, cyanobacteria and most algae and Euglena, and it is the process through out these organisms produce their energy (photoautotroph) [2]. In addition, oxygenic photosynthesis is the main event responsible for producing and keeping the atmospheric oxygen content ($\approx 20.95\%$).

In short, one can say that photosynthesis’ principal reactions involves the so-called water splitting (hydrolysis) reaction and the CO$_2$ fixation leading to production of carbohydrate compounds for long-term energy storage [3].

To drive the splitting, the oxygen-evolving complex makes use of the energy of four photons ($4 \text{hv}$), breaking two water molecules and recombining their atoms to form molecular oxygen (O$_2$), four protons (H$^+$), and four free electrons (e$^-$), equation 1.

$$2 \text{H}_2\text{O} \xrightarrow{4 \text{hv}} \text{O}_2 + 4 \text{e}^- + 4 \text{H}^+$$

(1)

Protons and electrons functions don’t cease after the water splitting reaction, by the contrary, they are the key players in other vital light-dependent reactions of great importance for the survival of photoautotrophic organisms.
transported to participate in the reduction of NADP$^+$ to NADPH [4]. The electron transport from PSII to NADP$^+$, although not intrinsically dependent, creates a differential of proton concentration between out- and inside of the thylakoid membrane which then is used for ATP production, which is known to be the molecular unit for energy trade on all living forms [5]. Taking in consideration the light-dependent and the non-light-dependent reactions, overall balance of photosynthesis can be described as,

$$6 \text{H}_2\text{O} + 6 \text{CO}_2 \longrightarrow 6 \text{O}_2 + \text{C}_6\text{H}_12\text{O}_6$$  \hspace{1cm} (2)

1.2. Thylakoid membrane, a complex apparatus

In plant cells, photosynthesis occurs in an organelle called chloroplast (Figure 1). Within the chloroplast, there are structures called thylakoids, which are nothing more than photosynthetic membranes that can exist in stacked and unstacked arrangements.

Since the first observation of chloroplasts under a light microscope, in 1837, by Hugo von Mohl [6]–[8], the structural complexity and anatomy of thylakoid membranes have been studied by modern techniques such as electron tomography [9] and cryo-electron microscopy [10]. These photosynthetic membranes are lipid bilayers with important protein complexes that participate in the light harvesting and first energy processes of photoautotrophic organisms, they are: Photosystem I, Photosystem II, Cytochrome b$_6$f,
and ATP synthase [4], [7], [11], [12]. Together they form the photosynthetic apparatus (Figure 2) which drives the electron transport from the oxygen-evolving complex to NADP$^+$ reduction and further production of ATP.

As in any chain, most light-dependent reactions will depend on each other, and light harvesting, performed by the PSI and PSII complexes, must be carried without interruption. The organization of the thylakoid membrane assures an efficient light harvesting setup. The 4 principal membrane complexes are orderly arranged in different parts of the membrane which we call appressed membrane, margins of the membrane, end membrane and stroma membrane. The first 3 regions of the membrane make up the stacked or grana membrane, and the stroma membrane, which extends through the thylakoid stroma to connect each membrane stack. On Figure 3, one can see that the linear electron chain is specifically organized with PSII in the appressed grana membranes and PSI in the non-appressed regions of the membrane. Additionally, there is almost no PSII on stroma membrane, making the ratio PSI:PSII very high on this region [13], [14].

Photosynthesis is also highly influenced by factors such as membrane packing and fluidity [15]–[22]. Therefore, lipids play a secondary but very important role in photosynthesis [23], [24]. The thylakoid membrane of *Arabidopsis thaliana* – the model organism used in this study – is mainly populated by four different lipids: MGDG ($\approx 50\%$), DGDG ($\approx 30\%$), SQDG ($\approx 5\text{-}12\%$), and PG ($\approx 5\text{-}12\%$) [25]. Each one of these lipids play a different role in the thylakoid membrane, and exert influence in photosynthesis in ways that are yet to be discovered [26]. This puzzling issue, opens space to arise several questions: Until what extend does lipid composition impact on protein diffusion along the membrane? Do membranes fully crowded with proteins impact on the diffusion of the highest mobile proteins? How is the interaction lipid-protein affecting the traffic on the thylakoid membrane?

*Figure 3* – (a), cross section of a thylakoid showing the different membrane regions including the appressed membrane, end membrane and lumen. (b), top-view of a thylakoid grana stack and representation of the interior composition and protein distribution of a grana disk [14].
1.3. Fluorescence a luminescent phenomenon

Fluorescence is part of a larger phenomenon called luminescence, which also comprises phosphorescence. Luminescence is the broadest concept for the emission of light from any substance, light that comes from the electronic transition of excited states. Fluorescence and phosphorescence differ in way the permit the electronic transition.

In singlet excited states, the spin of the electron promoted to a higher energy orbital is paired with the spin of the electron in the orbital of origin. As the transition to ground state is allowed by conservation of spin, this happens rapidly accompanied by the emission of a photon (Figure 4). The fluorescence emission rate is in the order of $10^8$ s$^{-1}$ and the lifetime of a fluorescent species usually ranges from 1 to 10 ns. Considering that the velocity of light in the void is approximately $3 \times 10^8$ m/s, it means that in terms of distance the light travels only 30 cm for 1 ns, the lifetime of some fluorophores. Thus, analysing the emissions of these species on single molecule level requires very sophisticated optical and electronic equipment.

The most common and fundamental way to show fluorescence spectral data of a compound comprises the light emission spectrum, a graph that shows the intensity of light emitted as a function of wavelength (nm) or wave number (cm$^{-1}$).

![Figure 4 – Jablonski's diagram, representation of electronic transitions: fluorescence is an electron transition phenomenon with broader variation of energy when compared to phosphorescence [58].](image-url)
1.4. Chlorophyll and Light Harvesting

Chlorophyll is a fluorophore, a molecule that absorbs light and then emits it in the form of fluorescence. Chlorophylls are compounds with a tetrapyrrole ring and a long hydrophobic hydrocarbon tail, the latter is known to be the responsible to anchor the molecule to the photosynthetic membrane. The tetrapyrrole ring binds a magnesium (II) ion, and it is the site of the electron arrangements of the photophysical processes which yield the final apparent green colour of this pigment as chlorophyll cannot absorb green light and thus reflect it.

In higher plants, there are mainly two types of chlorophyll, known as chlorophyll a and b, with a very small structural difference (Figure 5 (a)), a substitution of methyl group (Chl a) by an aldehyde group (Chl b). This small structural difference between Chl a and Chl b is enough to cause a difference in the absorption spectra of these molecules (Figure 5 (b)), making these pigments look to our eyes like blue-green and yellow-green colours, respectively.

The driving force of photosynthesis is the excitation of chlorophyll molecules. If the system would not receive external energy, the non-spontaneous hydrolysis reaction would never occur. When a chlorophyll absorbs a photon, it becomes excited, meaning that the electron distribution was destabilized and an electron is in a higher energy state. Chlorophylls can either absorb photons in the blue region of the visible electromagnetic spectrum (shorter wavelength, more energetic photon), or towards the red region of it (longer wavelength, less energetic photons). An electron which absorbs a photon in the blue region is extremely unstable and likely to rapidly lose energy to its surroundings in the form of heat, positioning itself in the lowest excited state, where it can be stable for a maximum of few decades of nanoseconds ($10^{-9}$ s) – a feature of any fluorophore called fluorescence lifetime. From the lowest excited state, four different possibilities might occur to the excited chlorophyll molecule:

1. It can return to its ground state by re-emitting a photon – fluorescence – with lower energy than the incident photon, once part of the energy is inevitably always lost through heat processes, yielding an emission in the far-red region of the visible spectrum.
2. The excited chlorophyll might return to its ground state by dissipating all its energy through heat, thereby no light is emitted.
3. An excited chlorophyll can transfer its energy to a close by non-excited chlorophyll molecule – excitation transfer through inductive resonance.
4. The energy of an excited chlorophyll molecule can be used in photochemical processes - meaning the conversion of light energy to chemical energy - by giving its absorbed energy so that a chemical reaction might occur.

These four different alternatives together comprise the fundamentals of light harvesting function and transfer processes which occur within the thylakoid membrane.
1.5. Light Harvesting Complex II and State Transitions

The major Light Harvesting Complex (LHCII, or M-LHCII) is a membranal trimeric protein responsible for light harvesting, acting as an antenna that can either be connected to PSI or PSII, depending on the received light intensity and quality (wavelength).

This protein is often considered to be the most abundant membrane protein on earth, binding half of the chlorophylls present in the thylakoid membrane [27]. The monomeric form of LHCII (232 amino acids) carries 8 chlorophyll a, 6 chlorophyll b, and 4 carotenoids (Figure 6 (b)) [27], [28]. Carotenoids participate poorly in light harvesting, but instead they have a crucial role on processes that protect the photosynthetic system: carotenoids quench chlorophyll triplet states preventing the formation of highly reactive single oxygen species [29]. Additionally they act as structural components of LHCs and are involved in photoprotective mechanisms of energy dissipation [30].

This protein in the trimeric conformation has a molecular weight about 25 kDa and has an bulk diameter about 75 Å [28]. The chlorophylls are distributed into two layers, one lying close to the stromal surface and the other near the luminal surface. They are disposed to have highly efficiency on energy transfer between them - with an average

![Diagram](image1)

**Figure 5** – (a), Common structure for chlorophyll molecules, where structural difference between Chl a and Chl b is evidenced. (b), the aldehyde substitution makes Chl b have its absorption maximum in more inner regions of the visible spectrum, when compared to its structural similar Chl a absorption.

**Figure 6** – (a), 3D structure of the trimeric LHCII from *Pisum sativum*. (b), structure of monomeric LHCII, represented with bound chlorophyll pigments. Molecular dinamic visual investigation based on structure from [59].
centre-to-centre distance between two neighbouring chlorophylls being about 11.26 Å [28] - and other pigments of surrounding proteins, such as the photosystems where the energy has its final destination.

The presence of non-bilayer lipids such as MGDG in such high abundance on the thylakoid membrane is only justified by its conjugation with the membrane proteins. Several studies show that MGDG has a high impact on stabilization of LHCII and DGDG lipids have been found crucial to establish interactions between different subunits of the PSII-LHCII super complex or upon monomeric or trimeric aggregation of several LHCs.

Under normal light conditions, LHCII is mainly connected with PSII in grana appressed membranes. However, plants are under constant changing light conditions, where intensity and incident wavelength are not always the same, thus absorbed light energy between photosystems needs to be balanced. This balance is obtained by a dynamic process with two defined conditions: the state 1 (S1), where the LHCII is mainly attached to PSII, and therefore mostly present in the grana membrane; and the state 2 (S2) where LHCII finds itself phosphorylated (LHCII-Pi) and at least partly attached to PSI present in the stroma membrane region. This balance and exchange between to changes occurs when the plastoquinone (PQ) pool - an intersystem electron carrier with additional function of monitoring changes in light quality and quantity, activates STN7 kinase to phosphorylate the LHCII. Upon phosphorylation, the LHCII dissociates from PSII, decreasing the light absorption on this photosystem, moves along the membrane and associates with PSI. This reorganization occurs on a timescale of minutes without any changes in gene expression. State transitions can be induced either by chemical manipulation of plastoquinone redox state (with inhibitors such as DCMU, or promoters like CCCP), or by controlled changes in light intensity or light quality.

With the introduction of the new phosphate group, the LHCII undergoes a conformational change to be able to dissociate from PSII. Effects of such conformational changes are not yet fully understood, especially if those enhance diffusional properties of the protein when it migrates along the thylakoid membrane. Until today, only two studies were performed with the focus to address diffusional parameters of LHCII on the thylakoid membrane, one using a technique called Single Particle Tracking, and another performed - on Chlamydomonas reinhardtii, an alga - by means of Fluorescence Correlation Spectroscopy (FCS). In the present work, FCS (see section 1.9. Fluorescence Correlation Spectroscopy) was used to address questions about LHCII mobility on the thylakoid membrane of higher plants, having as a biological model Arabidopsis thaliana.

1.6. Decay Time

Fluorescence is a temporally finite phenomenon, but its extensions is variable from species to species. Therefore, the time of fluorescence emission is a property with interest to study, since it can be, sometimes, given as a standard property of a given species on a specific environment. The decay time (τ, also called lifetime) is then defined as the average time that a fluorophore remains in the excited state following its excitation.

Being generally a first-order process, it can be described by kinetic law,

\[ n(t) = n_0 e^{-\frac{t}{\tau}} \]  

(3)
where \( n(t) \) is the number of fluorophore molecules in the excited state at time \( t \), \( n_0 \) is the number of molecules in the excited state at the instant of the excitation and \( \tau \) the lifetime. This is an exponential relationship since each fluorophore has the same probability of returning to the ground state. Experimentally the population \( n \) is measured in equipment in an arbitrary quantity called intensity \( I \). As such, it is common to observe the previous equation in the following form:

\[
I(t) = I_0 e^{-\frac{t}{\tau}}
\]  

(4)

Since the lifetime is defined as the average time that a species remains in the excited state before returning to the ground state, then it can also be calculated by a mathematical relationship between the rates of radiative and non-radiative processes,

\[
\tau = \frac{1}{k_r + k_{nr}}
\]  

(5)

1.7. Resolved measurements in time

Time resolved measurements are widely used in fluorescence spectroscopy because they provide a large amount of information that is not available when collecting steady state data from a molecule. These are divided into two methods: time domain (discussed in this paper) and frequency domain.

The time domain method is to excite the sample with a pulse of light of temporal width less than the decay time of the analysed molecule. The intensity \( I \) is measured, as a function of time, from the moment the light pulse is emitted; the decay time can be calculated through the slope of the decay curve of the graph log \( I(t) \), \(-\gamma\), as a function of time (Equation 6).

\[
-\gamma = -\frac{1}{\tau}
\]  

(6)

Fluorescence Lifetime Imaging Microscopy (FLIM) is a technique whose main foundation is fluorescence microscopy image measurements with lifetime information [31]. This technique has brought a great advantage to the branches of biochemistry and cell biology, providing solutions as fundamental as the monitoring of substances, essential or dangerous to cells, within certain cellular organelles. Also, other techniques based on time-resolved measurements are fundamental today in so many other areas, such as the case of time-correlated single photon counting.

1.8. Time Correlated Single Photon Counting

Time-Correlated Single Photon Counting (TCSPC) is a very powerful technique that relies on the excitation of the sample through pulses of light, spaced out for a well-defined amount of time (in the picosecond range, \( 10^{12} \) s) and in the detection of the emitted photons, considering the time interval between the excitation and the detection [32]. Each photon detected for each excitation cycle is stored in the graphical histogram per respective arrival time, ie the time difference between the excitation and the emission (Figure 7). The data collection grows and becomes consistent as multiple cycles are
accumulated, thus tracing a decay curve. The decay time, or lifetime, is one of the parameters that can be obtained with this curve, using slope $-\gamma$ to calculate $\tau$.

1.9. Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a technique developed in the early 1970’s, through the works of Madge, Elson & Webb (Cornell University, USA) [33]–[35], and Ehrenberg & Rigler (Karolinska Institute, Sweden) [36]. It is a minimally invasive diagnostic tool capable of addressing highly specific biological questions at the single molecule level [37]. The basis of the method is the statistical analysis of fluorescence signal fluctuations from emitting molecular species diffusing on a tiny confocal volume (0.1 - 1 fL). With appropriate models for different system dynamics, different parameters can be determined such as equilibrium kinetics, concentrations of fluorophores and diffusion coefficient (speed of diffusion) [38]. An FCS setup comprises the use of an inverted confocal microscope with one of the side ports for FCS detection (Figure 8). The detection setup is a complex system of optics with several lenses, mirrors, filters and pinhole with the aim to and restrict the emission signal to desired detection limits. On simple FCS, the excitation laser beam is directed via a dichroic mirror on the back-aperture of a water immersion objective with high numerical aperture (NA > 0.9). The same objective collects the red-shifted fluorescence emission from the sample, which passes through the dichroic mirror following to the pinhole and emission filters before reaching the detector (most commonly, an Avalanche Photon Diode detector). Most of FCS instruments have a TCSPC resolving unit attached, once its detectors have the time resolution to accomplish this technique, adding lifetime as an extra parameter to FCS experiments.
1.1. Aim of the diploma work

This project aims to address questions regarding LHCII mobility during state transitions. Making use of Fluorescence Correlation Spectroscopy technique and chlorophyll’s intrinsic fluorophore properties, the principal aim is to report the difference on diffusion coefficient of this highly mobile chlorophyll binding protein between state 1 and state 2.

Figure 8 – Simplified schematic of the main steps of single laser FCS measurement: fluorescence intensity is recorded for a small number of molecules in the very tiny detection volume (0.1 fL); the time-dependent fluorescence intensity, F(t), is analysed in terms of its temporal autocorrelation function, G(τ), according with mathematical models design to obtain desired parameters.
2. “All the light we cannot see”, a popular summary

2.1. Popular scientific summary

As both, a researcher in the field of plant biology, and nature lover, I have learned how intricate the topic of photosynthesis can be. It is actually difficult for me to fully understand what is more spectacular: the complexity of the photosynthetic processes as such, or the marvellous natural diversity initiated by the first photosynthetic organisms more than 3.2 billion years ago.

But was the first photosynthetic organism primarily responsible for shaping life as we know it today, or did light have the main role?

In the beginning of Universe’s existence, about 13.8 billion years ago, the first photons were formed, allowing the Cosmos to shine and fill up with amazing colours that today we gaze when looking the sky with our own eyes. This seems to be a beautifully simple story, but light is indeed a complex physical phenomenon, which results from the excitation and relaxation of atomic particles called electrons. Light can be emitted in specific form of light which physicists named as fluorescence. The study of light as fluorescence has extended the horizons of chemistry and biology, revealing new with the introduction of new and revolutionary scientific techniques.

The purpose of the current study is to explain the involvement of light – under the form of fluorescence – in the photosynthetic processes, with the focus on the diffusion of the LHCII protein present on the thylakoid membrane (inside plant cells). This protein is responsible for capturing the major part of the absorbed light that plants receive from the sun. Modifications occur in this protein when the light quality and intensity changes, as for instance when a cloud passes in the sky and leaves the plant in the shadow. To compensate for these changes, modifications occur as a response of the plant to continue performing efficient light harvesting in the altered conditions. Such environmental changes signal and modify the LHCII with an additional phosphate group (phosphorylation), which makes the protein move between two regions of the thylakoid membrane.

How does this protein move? How fast does it move? Is it moving at a different speed depending on the phosphorylation state? What structural changes are happening to the protein when it is phosphorylated? Does the LHCII protein interact with other elements in the thylakoid membrane (e.g., lipids) when it is moving? Are those interactions different depending on whether the protein is phosphorylated or not?

These questions have been addressed by scientists for a while and yet no good answers to these questions were found. This paper presents a study done with Fluorescence Correlation Spectroscopy (FCS), a modern technique, with the attempt to answer some of these intriguing questions.

Photosynthetic processes and the structural organisation of the thylakoid membrane are an incredible proof of how Nature tries to be perfected, and this assures an efficient sunlight harvesting arrangement with a flexibility to adapt to a broad range of environmental conditions. Scientists try to understand these intricate phenomena with the hope to extend even more plants’ resistance to different environmental factors, and/or to increase biomass production, aiming to increase production of food or energy. These goals are of high importance to humankind once they can help to fight against food shortage in poor regions of our mother Earth, and more green-friendly means of energy production.
2.2. Social and ethical aspects

Photosynthesis has global consequences for our climate, bioenergy resources, and agriculture. Growing in silence, plants have a substantial impact in our lives, even when we do not notice them.

Material science and engineering try to develop new materials to increase efficiency of solar panels – pushing forward the use of renewable energies – in the same way plant biology aims to understand the functioning of plants to increase their efficiency and biomass production (one of many goals) [39]. In other words, the scientific community in the field of plant biology aims to give its social contribution by understanding and helping Nature to accomplish its status of perfection.

Much is known about the fundamental units comprising diverse photosynthetic organisms, but much less is known about the organismal level of regulation that controls the efficiency of solar conversion, water consumption and nutrient utilization. How can energy be dissipated to prevent damage? Or even better, how can the energy excess be used, saving plants from damages and making photosynthesis a fully efficient process? Understanding how photosynthetic complexes are assembled and maintained at a fundamental level will enable us to improve crop growth and crop protection in specific climates. However, some can see this work as basic research projects that primarily will end up in textbooks, having no applicable consequence in our lives. Many forget that science is based on a tree-like structure, where the foundational concepts are the basis – the trunk – that sustains and gives origin to diverse ingenious and useful products – the leaves – for our daily lives. Without the trunk, there are no leaves.

In our world about 100 000 people per day die of starvation or malnutrition [40]–[42], which accounts for a large number when compared with deaths by different types of cancer (≈ 25 000, [43]) and malaria (≈ 1 300, [44]). To cope with the biggest worldwide problems, such as malnutrition, scientists use genetic modified organisms (GMO). There are cases of success from the scientific point of view, such as the worldwide famous “Golden Rice” [45], rice that has been transformed with a vitamin A producing gene from sunflower with the aim to overcome lack of vitamin A intake that leads to blindness in children. The European Union has been focused on creating GMO legislation [46], and has spent a large amount of money to investigate if GMO could lead to health issues, yet did not find reasons to be concerned [47]. As a scientist, it is my duty to play a social role for advocating correct science, its benefits, as well as the associated risks and problems.

From another ethical point of view, the field of plant biology has the advantage of almost only using plants as specimens in the conducted research, which avoids many negative ethical aspects. On the other hand, in other research fields, the ethical issue of performing experiments on live animals – a fact that attracts the attention of many activists - needs to be seriously considered.
3. Experimental

3.1. Methods

**Strains and Growth Conditions.** *Arabidopsis thaliana* wild type (ecotype Colombia-0) strain and STN7 and STN8 mutant strains seeds (a kindly gift from professor Eva-Mari Aro, Turku University) were sterilized, plated on Murashige & Skoog media plates, stratified for 2 days at 4°C (in darkness) and then moved to short day conditions (8h light/ 16h darkness) in a growth chamber. After 14 days plants were transferred to soil (soil-vermiculite mix) and kept under short day conditions until approximately 10 weeks of age. Plants were watered regularly in a 3 day rhythm. Once a week nematodes were applied. The light intensity in the chamber had a continuously photon flux density of 120 µmol m⁻² s⁻¹, temperatures were kept between 20 – 22 ºC, and humidity above 50%, corresponding to typical normal conditions for optimal chamber growth.

**Chloroplast and Thylakoid Extraction and Isolation.** Freshly harvested *Arabidopsis thaliana* leaves were jointly ground with cold Grind Buffer (20 mM Tricine pH 8.4, 450 mM C₆H₁₄O₆, 10 mM Na-EDTA pH 8, 5 mM NaCl, 5 mM MgCl₂) to where 2 g of bovine serum albumin (BSA) and 2 g of C₆H₇O₆Na were freshly mixed, and the preparation was filtered through a 4 layer of Miracloth (20 μm pore size) and centrifuged at 1000 g for 3 min. Pellet was gently resuspended in cold Wash Buffer (20 mM Tricine pH 7.6, 330 mM C₆H₁₄O₆, 5 mM MgCl₂). At this step intact chloroplasts were collected for FLIM experiments. Most of the sample proceeded to thylakoid isolation by 1000 g centrifugation for 3 minutes. Pellet collected and resuspended in cold Shock Buffer (20 mM Tricine pH 7.6, 5 mM MgCl₂) and centrifuged again at 200 g for 5 minutes. Final pellet was collected and resuspended in cold Stack Buffer (20 mM MES pH 6.3, 5 mM MgCl₂, 15 mM NaCl) for appropriate concentrations to be used in the future experiments.

Protocol based on the first steps of the traditional BBY (Berthold, Babcock and Yocum, 1981) membrane preparation protocol [48], with minor changes developed by Arellano, J. B. *et al.* (1994) [49]. The extraction was performed in cold room conditions and samples were preserved in ice for most of the process.

**Thylakoid Membrane Fractionation.** 5 mL of 0.8 mg Chl/mL thylakoid suspension were mixed with 5 mL of 0.4% digitonin solution for about 2 minutes (at room temperature), allowing membrane solubilisation. Solubilisation was terminated by addition of 90 mL cold buffer (100 mM C₁₂H₂₂O₁₁, 10 mM NaPB pH 7.4, 5 mM NaCl, and 5 mM MgCl₂) and sample centrifuged in 3 steps with washing steps with previously indicated buffer: 10,000 g for 15 minutes, proceeding with supernatant, 40,000 g centrifugation for 30 minutes; pellet was collected after final ultra-centrifugation at 100,000 g, for 60 minutes. The final sample was diluted in 25 mM MES (pH 6.5) cold buffer solution to a concentration of 0.25 mg Chl/mL, before storage at -80°C.

This experiment was performed in cold room conditions, except when counter-indicated, and samples were preserved in ice for most of the process.

**Isolation of LHCII.** Isolation of LHCII was conducted for each strain, starting from 0.8 mg Chl/mL solution of freshly extracted thylakoids, buffered at pH 7.5. Two different batches of wild type thylakoids were prepared to induce state transitions during an
incubation time of 30 minutes. Incubation to induce S1 was performed in dark condition with addition of 10 μM DCMU, and simple white light incubation (photon flux density 11.2 μmol m⁻² s⁻¹) was used to induce S2. To dissolve the membranes, a second incubation step of 30 minutes was performed with addition of 0.8% (w/v) Triton X-100, continuous stirring.

Linear sucrose density gradient tubes (0.1M to 0.8M) were prepared in advance by freeze-thaw method: two sucrose solutions with 0.1M and 0.8 M concentrations were prepared, containing 0.02% Triton X-100 and 2mM tricine pH 7.5. Mixed solutions with 1:2 and 2:1 ratios were prepared from the two freshly prepared sucrose solutions. On 38.5 mL Ultra-Clear™ thinwall ultracentrifugation tubes (Beckman Coulter, USA), 7.5 mL of each of the four sucrose solutions were added by decreasing order of sucrose concentration and temporally spaced by, at least, 100 minutes of freezing. Several hours prior to the isolation experiment, sucrose tubes were thawed slowly at 4°C, allowing the formation of linear sucrose gradient.

8.5 mL of sample were carefully loaded on top of each sucrose gradient and ultracentrifugation was performed on Optima L-90K (Beckman Coulter, USA) with a SW28 Ti swing-out rotor (Beckman Coulter, USA), at 28000 rpm for 12 hours, 4°C.

From each tube, the correspondent LHCII band was collected and poured out to a beaker and sample was diluted in milliQ water and pH adjusted to 7.1, at normal room temperature. KCl was added until a final concentration of 300mM, followed by 15 minutes of incubation, continuous stirring. Centrifugation steps of 5 minutes were performed at 2500 rpm, immediately washing samples with 100mM KCl and finally 25 mM MES (pH 6.5) cold buffer solution. All the experiment was performed in cold room conditions, except when counter-indicated.

**Chlorophyll and Protein Quantitation.** For quick and rough estimate of protein quantitation, and quality assessment of membrane fractionation, spectroscopic determination method according to Porra et al. (1989) [50] was used.

**Protein Assessment and Phosphorylation analysis.** Self-casted SDS-PAGE (17.5% 37:1 Acrylamide/bis with 6M CH₃N₂O) stained with Coomassie Blue was the elected method for protein assessment based on molecular weight. For phosphorylation analysis, western-blotting was implemented using a phospho-threonine primary antibody (Cell Signaling TECHNOLOGY, USA), 1:1000 dilution, on Amersham WB System (GE, United Kingdom).

When western-blotting was performed, an SDS-PAGE gel with Cy5 pre-labelled proteins ran on the same Amersham WB System. All reagents used belong to the provided Amersham WB kit.

**Oxygen Evolution.** As an indicative method to assess photosynthesis efficiency, oxygen evolution measurements were performed using Oxygraph (Hansatech, United Kingdom), and a silver (anode) – platinum (cathode) electrode bathed in saturated KCl solution. For every experiment the measuring cell was loaded with 20 μg sample (chlorophyll based measurement) and sample was buffered with 500μL of measuring buffer (40 mM MES pH 6.5, 400 mM Sucrose, 10 mM NaCl, 5 mM MgCl₂) and 40μL of 40 mM PPBQ (electron acceptor) were supplied and the measuring cell filled up to 1 mL with milliQ water. Measurements at constant 20 °C were performed for four independent replicates of each strain.
**PAM measurements.** Chlorophyll fluorescence studies to characterize each strain were performed by use of Pulse Modulated Amplitude, on PAM-210 (Walz, Germany). Plants were dark-adapted for at least 30 minutes prior to the experiment. The total measuring time was of 120 s, with saturation pulses (width = 800 ms) and data collected every 10 s. Measurements were performed on 4 independent replicates (different leaves), for each 5-independent biological (different plants, 8 weeks old), making a population of n = 20 for statistical purposes.

**Lipid Extraction and Isolation.** 5 g of freshly harvested Arabidopsis thaliana wild type leaves were blended and rapidly submerged in boiling 80% isopropanol solution at 80°C for 10 minutes. To the boiling solution was added a mixed solution of 0.73% NaCl, methanol and chloroform (1 : 3.5 : 7, respectively). The mix was toughly blended and then poured into a Büchner funnel. The ground leaves were washed out 3 times with a H$_2$O - CH$_3$OH - CHCl$_3$ (1 : 4 : 2) solution. A large volume of mix solution of chloroform and 0.73% NaCl (1:1) was added to the lipid extract before collecting the chloroform phase (shook and separated with a separatory funnel). The chloroform phase was evaporated and obtained dry lipids were diluted in chloroform to a desired volume to load on TLC plates. Lipids were loaded and separated by Thin Layer Chromatography (20 x 20) cm silica gel 60 plates (Merck, Germany), previously activated (110°C, 1.5 h, and left overnight in desiccator). One dimensional TLC was performed, developed in CHCl$_3$ - CH$_3$OH - H$_2$O solution (75 : 25 : 2.5) and revealed with iodine. MGDG and DGDG bands were scratch out of the plate, and lipids washed out of silica powder with chloroform. Thin Layer Chromatography was used as both a separation and assessment method.

**Fluorescence Correlation Spectroscopy on Stroma Membrane.** The bottom surface of a Nunc™ Lab-Tek™ 8-well chambered cover glass (Thermo Fisher Scientific, USA) was covered by 40 μL of adsorption buffer (10 mM Tris pH 7.3, 150 mM KCl, 20 mM MgCl$_2$). Then, 20 μL of freshly prepared thylakoid membranes (0.25 mg Chl/mL, in MES buffer pH 6.5) were added to the adsorption buffer on the cover glass surface and incubated for 10 min. The surface was gently rinsed with 500 μL of MilliQ water once and then covered with 40 μL of recording buffer (10 mM Tris, pH 7.3, 150 mM KCl). The membranes attached to the cover glass surface were observed by using an in-house built setup based in Abberior (Abberior Instruments, Germany) detector solution, TCSPC acquisition card modules by Becker & Hickl (Germany), and IX73 confocal laser scanning microscope (OLYMPUS, USA). Chl pigments in the isolated membranes were excited at 633 nm with a pulsed laser (2 μW power) through a water immersion objective lens UPLSAPO Super Apochromat 60XW (60x, 1.2 NA, OLYMPUS), and the emission was detected through a 650- nm long pass filter. The confocal pinhole diameter was adjusted to minimize the confocal detection volume. The excitation laser power was adjusted so that high enough signal-to-noise ratio was obtained for diffusion analysis, and the photobleaching of Chl pigments was also minimized. For imaging control the Imspector software (Abberior Instruments, Germany) was used and data acquisition collected by SPCM software (Becker & Hickl, Germany). Lab-Tek™ 8-well chambered cover glasses were plasma cleaned by Zepto Plasma System (diener electronic, Germany), to offer optimal membrane adsorption to the glass.

**Fluorescence Correlation Spectroscopy on GUV.** GUVs were prepared by my close collaborator Johannes Sjöholm (Stockholm, Albanova, KTH/SU), using an electroformation protocol. The protein of interest is inserted in the liposome vesicle membranes.
by mixing with 1 μL of 0.5 μM solution of purified LHCII, allowing to incubate for 30 minutes. The bottom surface of a Nunc™ Lab-Tek™ 8-well chambered cover glass (Thermo Fisher Scientific, USA) was coated with 150 μL of 0.1 mg/mL BSA-Biotin solution and incubated for 10 minutes. After BSA-Biotin removal, 150 μL of 0.025 mg/mL NeutrAvidin® were added to the glass surface and incubated for 10 minutes. The glass surface was rinsed with milliQ water and the 20 μL solution of prepared GUVs were finally added to each well with 380 μL of measuring buffer (10 mM PBS pH 7.4, 2 mM KCl, 100 mM C₆H₁₂O₆). Measurements were performed in the same setup described previously. A total of 15 measurements on different GUVs for each strain were performed, with 120 s measuring time. For all experiments, the control measurement in solution near the analysed membrane was also done to confirm no leak of CBPs from membranes into the buffer solution.

**FCS data analysis.** FCS measurements of the CBPs in the membranes and the data analysis were done using the software described above. Briefly, Chl FAFs (Fluorescence Autocorrelation Function, G(t)) were calculated as:

\[
G(t) = 1 + \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} \tag{7}
\]

where \(\tau\), \(I(t)\), indicate time delay and fluorescence intensity, respectively. The acquired \(G(t)\) values were fitted using a one-component diffusion model:

\[
G(t) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{\omega^2 \tau_D}\right)^{-1/2} \tag{8}
\]

where \(\tau_D\) is the diffusion time; \(N\) is the average number of Chl fluorescent molecules existed in the excited confocal detection volume defined by the radius \(\omega = 0.271 \, \mu\text{m}^2\), a parameter known and associated with calibration done on the used system) and the length which is related to FWHM of the gaussian profile of the used laser. The diffusion time, \(\tau\), is related to the translational diffusion coefficient, \(D\), by

\[
\tau = \frac{\omega^2}{4 D} \tag{9}
\]

All measured Chl FAFs were fit with the *Pulsed Interleaved Excitation Analysis with MATLAB* script developed by Müller, B.K (2015) to use on MATLAB software (The MathWorks, USA), using the model described above. Triplet and blinking terms were excluded from the fitting. For all experiments, the control measurement in solution near the analysed membrane was also done to confirm no leak of CBPs from membranes into the buffer solution.

**Fluorescence Lifetime Imaging Microscopy.** Measurements were performed on IX83 confocal laser scanning microscope (Olympus, USA) in association with a full detection setup by PicoQuant (Germany). Excitation wavelength of 640 nm (PicoQuant LDH-D-C-640 laser [≤ 90 ps pulse width, at FWHM]) and emission collected in the 650 - 670 nm range. Symphotime (PicoQuant, Germany) software was used for data acquisition and data analysis. Lifetimes were extracted by tail fit of obtained TCSPC curve with 4
component lifetimes. Lifetime obtained by imaging was calculated on a pixel base and averaged by amplitude.

FLIM and FCS techniques were made available through our collaborator Jerker Widengren at KTH (Albanova complex, in Stockholm, Sweden).
4. Results and Discussion

In this work 3 different strains of *Arabidopsis thaliana* ecotype Colombia-0 were used: Wild type, the strain that prevails among individuals in natural conditions; STN7, a mutant with stn7 protein gene knockout; STN8, a mutant with stn8 protein gene knockout. STN7 is known to be the main responsible protein to phosphorylate LHCII and STN8 is a protein known to phosphorylate primarily N-terminal threonine residues of psbA/D1, psbD/D2, psbC/CP43 and psbH, proteins that belong to PSII core [51]. The aim is to investigate the influence of phosphorylation of PSII-LHCII complex on different biophysical properties, comparing the impact of phosphorylation at PSII core level (STN8) and LHCII level (STN7) with the natural performance of wild type strains.

4.1. Characterization of the biological samples

Under normal light and temperature conditions STN7 and STN8 strains have a comparable growth to wild type plants, presenting any phenotype differences (see Appendix 1 – Photography of experimental setups).

To address the quality and characterize the biological samples to be used in this work, PAM measurements were performed on 8 weeks’ age plants for each grown strain. Light curve PAM measurements (*Figure 9*) give an idea about the performance of the photosystems’ efficiency and energy loss in processes such as heat loss. Once that single PAM was performed, only efficiency of photosystem II is address, Y(II).

As anticipated, the wild type strain presents a higher quantum yield of photochemical energy conversion, when compared with the mutants that lack the kinases STN7 and STN8. STN8 is the mutant with the lowest photochemical energy conversion and the highest energy loss in PSII, demonstrating that phosphorylation N-terminal threonine residues of PSII core proteins by STN8 kinase plays an important role to keep functional photochemical energy conversion at the photosystem core. \(F_v/F_m\) values compare the dark-adapted fluorescence state, \(F_v\), to maximum fluorescence, \(F_m\), a state where the maximum amount of PSII reaction centres were closed by light saturation. A decrease of \(F_v/F_m\) values between WT (0.789 ± 0.012) and STN8 (0.758 ± 0.016) indicates that the mutation affects PSII in a dark-adapted state. STN7 (0.801 ± 0.014) shows no alteration in the maximum quantum yield of PSII, since the photosystem core is not affected by the mutation. These results had shown to be statistically significant (see Appendix 2 – Data Analysis).

*Figure 9* – Light curves for each strain were plotted according to quantum yield of photochemical energy conversion in PS II, Y(II), and quantum yield of regulated non-photochemical energy loss in PS II, Y(NPQ), left and right, respectively. Results averaged from a population of 20 replicates (4 independent leaf measurements per each 5 biologically independent replicates, \(n = 4 \times 5 = 20\)).
From the plants analysed by PAM, intact chloroplasts were isolated, observed and imaged using a confocal microscope with FLIM unit (**Figure 10**). Wild type chloroplasts reveal to have a predominant shorter lifetime, under 1 ns, when compared with the mutants. In comparison, most of the inspected STN8 chloroplasts have longer lifetimes, between 1 to 2 ns, with some individual objects having lifetimes shorter than 1 ns, while STN7 mutant organelles presents a broader and very distinctive lifetime range between 1 and 4.5 ns, approximately. The analysed amplitude averaged lifetime for each strain were determined: 0.657 ± 0.002 ns (WT), 1.237 ± 0.003 ns (STN7), 0.904 ± 0.002 ns (STN8). This is new FLIM data, reported for the first time on intact chloroplast of the selected strains. From my interpretation, phosphorylation of the LHCII induces small conformational changes on the structure of this protein which are significant enough to change the distances between chlorophylls and interfere with mechanisms of energy transfer among them.

Extracted thylakoids membranes from each strain were subjected to oxygen evolution measurements under common light conditions (**Figure 11**), with the aim to report the photosynthetic efficiency of the mutants by comparison with the wild type strain. Mutants have a decrease in efficiency of 30% at least, with STN8 showing a drop of about 40%.
The newly reported photosynthetic efficiency rates given by oxygen evolution measurements support the results of PAM, demonstrating that STN8 is the poorest mutant performing photosynthesis.

4.2. Diffusional study of LHCII on stroma thylakoid membrane

Thylakoid membranes were fractionated by solubilizing the membrane with digitonin detergent. Stepwise centrifugation was performed to allow separation of grana and stroma membrane fractions. Both fractions were assessed by visualizing the present proteins on SDS-PAGE (Figure 12 (a)). To support the visual classification, Chl a/b ratios were measured on stroma (4.56 ± 0.51) and grana (2.51 ± 0.06) membranes. The comparison with published data regarding the distribution of protein complexes on both types of membranes [11], [13], [14], supported that a good separation of stroma membrane had been achieved. When it comes to the FCS measurements of LHCII diffusion on thylakoid membranes from higher plants this was not been done before, or at least published, which made this part of the work especially elaborative. The protocol comprised a photobleaching step prior to the real measurement with the aim to eliminate the fluorescence signal coming from chlorophyll binding proteins (CBP) others than the highly mobile LHCII (Figure 12 (b)). By principle, if any diffusion would be detected in the photobleached region, it would be due to the re-entry of LHCII proteins in the area. Measurements started on the photobleached region of the membrane, but no diffusion was ever detected, and therefore any data with potential to be analysed could be collected.

By inspection of different confocal microscopy images of the sample, I found that the prepared membranes had a size which is too large to be stroma membranes in its natural physiognomy. This could be due to possible aggregation which could explain the observations, and several attempts to reverse the aggregation were attempt, but no profitable outcome ever came from FCS measurements. Despite the several attempts made to reverse the aggregation – use of detergents, change of buffers, and ultrasonication protocols – no profitable outcome ever came from FCS measurements.

Photobleaching at given laser power (≈ 2 μW) should not destroy the proteins, but rather its fluorophores only. One can then speculate that being unable to detect fluorescence fluctuations after photobleaching can also be a consequence of the photobleached LHCII
proteins still being there, and thus there is no necessity for new LHCII to come into that region.

4.3. Diffusional study of LHCII on Giant Unilamellar Vesicle membranes

The main idea of the work changed from *ex vivo* to *in vitro* observations of LHCII integrated on artificial liposome membranes.

LHCII was isolated from the thylakoid membrane by solubilisation with triton X-100 detergent and isolated by means of ultracentrifugation on sucrose density gradient solutions. The collected LHCII fractions were clearly enriched with LHCII, which could be seen by SDS-PAGE (*Figure 13*). This was further established by Western-Blotting performed with a phospho-threonine primary antibody revealing phosphorylation of the LHCII band (25 kDa) on every lane. Additionally, a band between 50 and 66 kDa size was found to identify a highly-phosphorylated protein for WT S1 and STN7. From the known phosphorylated protein complexes with such high molecular weight, one can expect that it is ATP synthase β-subunit (F-ATPase β, 54kDa) [52]. From the present immunoblot analysis, one can speculate that state transitions influence the phosphorylation of ATP synthase β-subunit which is notorious on WT in state 1 and STN7, but silenced on STN8 mutant, as previously reported [53].

*Figure 12* – a) SDS-PAGE results of the two isolated types of thylakoid membrane. Samples were loaded with a concentration of 0.45μg Chl/μL. b) Images of isolated stroma membrane before (left) and after photobleaching (right).

*Figure 13* – On the left, SDS-PAGE protein separation with pre-labelled proteins with Cy5 dye reagent. On WT S1 and WT S2 lanes 0.6 μg of samples were loaded and 1.2 and 1.6 μg for STN7 and STN8 lanes, respectively. To the left, results from Western-Blotting from the same gel.
Unfortunately, due to mistakes done when loading the gel, one cannot evaluate the phosphorylation levels between LHCII proteins from different preparations, and the software cannot accurately reach a ratio of total protein to total phosphorylated-protein content.

Each of these groups of samples proceeded to be tested by FCS. After a successful proof of concept that fluorescence fluctuations at the surface of GUV membranes – made with DOPC lipids – could be detected, it was decided that later production of GUVs would comprise a more natural lipid composition. MGDG and DGDG lipids where extracted from wild type Arabidopsis thaliana with 10 weeks of age and separated by Thin Layer Chromatography (see Appendix 1 – Photography of experimental setups). At KTH, production of GUV with different lipid compositions was successfully achieved and preparations with LHCII isolated from each different strain were implemented.

The possibility to test a different range of GUV lipid composition was compromised not only due to limited experimental amount of time, but also due to intrinsic characteristics of lipids that would yield a good number of produced GUVs. To be on the safe side, I was obliged to keep always some concentration of the artificial but very stable DOPC lipid, thus defining the following 4 classes of produced GUVs: DOPC 85% - DGDG 15%, DOPC 55% - DGDG 45%, DOPC 40% - DGDG 45% - MGDG 15%, DOPC 40% - DGDG 45% - MGDG 30%. MGDG is known to be a single layer lipid and for that reason, besides being the most abundant lipid on the thylakoid membrane, produced GUVs had no more than 30% of it in their composition.

Figure 14, summarizes the averaged results after extensive FCS data analysis. Additionally, the same figure presents lifetime data collected from a TCSPC unit coupled to the used FCS instrument. Lifetimes were tail fitted with a single exponential decay meaning that, even if more complex, the photophysics of the present fluorophores were simplified to one single component lifetime.

Based on averaged results, only LHCII isolated from STN8 mutant seems to have a direct correlation between diffusion coefficient and lifetime, both decreasing upon introduction of MGDG lipids on the membrane.

Regarding the measurements of diffusion coefficient, I do not consider to have reached significant results, or at least detected a pattern between samples that could help me to give answer to the aimed question “has LHCII different diffusional properties, such as different diffusion coefficient depending on is level of phosphorylation?” Also, because of the limited diversity of GUV composition, there is no room to assert existing tendencies for diffusion coefficient with the variations on lipid composition.

Most likely, the factor which makes such data of difficult analysis is the very small difference on diffusional speed that, was expected to be observed between phosphorylated and un-phosphorylated protein. Three different matters help me to explain the statement above: once we are talking about diffusional properties that might change within a protein that keeps its tertiary structure and therefore obtained results would always be within the same order of magnitude; on higher plants, the percentage phosphorylated LHCII on the thylakoid membrane (mainly on stroma regions) is known to only increase a few, 10 – 20 %, compared with 40 % phosphorylated LHCII on algae [54]; last but not least, the sample preparations need to be more carefully executed and a good western blotting analysis is a must to confirm actual differences on phosphorylation level between samples.
**Figure 14** – Averaged diffusion coefficient and lifetimes (of 10 independent GUVs) from 16 different experiments (4 LHCII preparations x 4 GUV preparations) are presented, 10 independent measurements per experiment (n = 10). Error bars indicate standard deviation.
5. Conclusions

This work aimed to answer if phosphorylation of LHCII, as it is taking place in state transitions, exercises influence in its mobility. With such a simple question one might imagine a quite direct and straightforward project plan. However, it turned out that some parts were not as straightforward as expected. Also working with higher plants that need 8 weeks to fully grow to an adult plant stage makes the task even more challenging with respect to planning. Another challenging factor was that FCS and FLIM measurements were performed at KTH in Stockholm, which needed further planning and coordination, as well as subjecting samples to longer storage periods, compromising their freshness. These are some of the problems that hindered me from obtaining clear results with the possibility to answer the questions addressed in this project. However, I realized that mistakes made early in the project have led to inconclusive results in final experiments, and therefore, in the following paragraphs I summarize those mistakes from which I learned.

Firstly, this work was designed to address diffusional parameters of LHCII on ex vivo thylakoid membrane observations. Unfortunately, when performing FCS, no diffusion could be detected on the membrane. One of the following propositions might explain what occurred:

1. The isolated membranes are not real stroma membranes and more elucidative methods to assess membrane physiognomy, such as atomic force microscopy, could be used for the purpose.
2. If membrane fractionation and isolation of stroma membranes was accomplished with success, regarding FCS measurements, the problem most likely resides in sample treatment, rather than in the photo bleaching procedure that precedes the measurement. For this analysis, the use of freshly extracted thylakoid membranes, the concentration and media at which samples are kept might seriously condition the outcome of the experience.
3. The migration of LHCII in higher plants is not a reality, instead the decreasing of LHCII on grana membrane and the enrichment of LHCII on stroma membrane maybe be explained by other mechanisms such as the introduction of newly synthesized LHCII-P on stroma membranes. This however, would challenge the common view within the field.

The proposition advanced in point number two was taken as the most likely explanation, and thus shaped the adopted experimental course which lead to several hours attempting to revert aggregation of stroma membranes. Different detergent concentrations were used, combined with ultrasonication protocols, which never achieved experimental success by FCS. Therefore, I concluded that trying to revert aggregation was not a solution.

From FCS measurements done on GUV membranes, no significant conclusions can be taken once no strong pattern was found neither among the same strain on GUVs with different composition, nor within different strains for a specific GUV composition. Unfortunately, this still leaves open the main question aimed to be addressed in the present work. Available time for measurements and nature/quality of prepared samples were the major limiting hindrances at this stage.

There was an effort to integrate other experiments that were performed using other spectroscopic techniques with the intention to support and elucidate the photophysical behaviour variances of different LHCII preparations, which by distinct reasons never made it to figure in document. Among those I want to mention: the collected absorption spectra
which consistently showed that LHCII isolated from wild type induced to state 2 and STN8 have higher absorption efficiency at more energetic wavelengths by comparison with the ones isolated from wildtype S1 and STN7; and the steady-state fluorescence measurements which revealed an interesting wavelength shift of 2 nm between wild type and both mutant preparations.

The lack of new biological material, associated with the time limited project, did not enable to repeat experiments with newly improved preparations, neither to discuss nor confront the obtained results with available literature. Additionally, I learned that performing experiments on biological material coming from different batches, or biological material with different age, does not help to establish nor to reinforce conclusions between experiments.
6. Outlook

Having the chance to correct myself, and especially after starting to master the techniques required to accomplish this project, I would attempt to repeat all the experiments starting from only one unique batch of biological objects.

If returning to ex vivo FCS measurements, the stability of stroma thylakoid membranes must be seriously investigated. After membrane isolation solutions with different sample concentrations must be left to incubate and aggregation can be followed by confocal microscopy. Diverse preparations of final storage buffer, with variation of sugar and detergent concentration, as well as ionic strength, must be considered and aggregation followed as suggested above. In this way, the preferable ex vivo FCS measurements would be considered worthy to try again.

In the future, isolation of LHCII should be performed more meticulously, keeping the right detergent to protein ratio under control, and well defined conditions for induction of state transitions. We have now available in our lab CCCP, a promoter of state transitions 2, which can soon be used in this experiment. Addition of cyanide – a chemical known to have a lock effect on state transitions [55] – before solubilisation of the membrane can be applied. Accomplishing the mentioned can guarantee better preparations and more clear results on future in vitro FCS experiments.

The range of sucrose density gradient can be narrowed to allow better separation of trimeric LHCII from other proteins, yielding a better isolation. Electrophoretic and immunoblotting techniques (now finally functional again in our laboratory) can be repeated to address accurate phosphorylation levels for each strain. The same amount of protein should be loaded for each prepared sample and 3 different quantities of each sample can be loaded to help concluding about the purity of isolation. Additionally, better purification of LHCII can be also attempted by using chromatographic techniques such as affinity chromatography, and the protein quantitation determined by the RCD™ protein assay (BIO-RAD, USA).

There is a chance to get a FCS unit coupled with the new confocal microscope that is about to be bought for in-house purposes, facilitating organisation and planning complexity, thus consisting of a good opportunity to review the present study.

Regarding other possible future projects, I will mention some of the ideas that I came to realize. Thorough investigation of the mechanisms of energy charge transfer within fluorophores of phosphorylated and non-phosphorylated LHCII can be considerate to be a feasible future project, once new Förster Resonance Energy Transfer (FRET) equipment will be soon available in our department. Packing and protein density of the thylakoid membrane, how much does it differ from the GUV scenario? How are protein-lipid interactions influencing chlorophyll lifetime of LHCII and other chlorophyll binding proteins? These are some of the questions that I am ambitious to address, which will bring new knowledge and might support answers tried to be address in the current project.
Acknowledgement

Dear Wolfgang, thank you for having me during this wonderful year! I’m super grateful that you trusted in me, and in my capacities, to pursue my dreams and work with the techniques and project that I dreamed of. Our connection is not only merely academic, but definitely a good friendship!

Lennart Johansson, kind in spirit, kind to help. Thank you for the provided knowledge about photochemistry, I’m sure it will continue to prove to be helpful in the future.

Eva Selstam, for your tenderness, for all the inspiration I found on it, and your important knowledge about lipids, I’m very thankful. I also appreciated a lot that you borrowed me the necessary material to do the lipid extraction.

Jerker Widengren, thank you so much for opening the doors of your amazing facilities at AlbaNova, which without access to it, this work would never be possible. I thank you also all the support and interest that you put on the developments of my project and for believing in this collaboration.

Johannes Sjöholm, you have been my right arm in this adventure of connecting my works/worlds in Umeå with yours in Stockholm. Thank you for all the time you spent with me around the microscope, preparations, data analysis and discussion. Thank you also for suffering together with me the misadventures of this project, and for proportionating an alternative analysis of LHCII diffusion on GUV membranes.

Kati, wonderful “gardening teacher” and friend. With your gentle spirit you thought me to have the most delicate of the cares for Arabidopsis. I’m very thankful! I miss you already so much… Come back soon!

Laxmi, you were my office companion since the first day until the last one, always present for anything needed, or just for your silent presence while seated on the next desk. Thank you my dear.

Lorenza, Marina, Raik, Zivan and Amit, from you I have been learning many things that already helped me to mature in my relationship with others. Thank you for all the smiles you gave me, for all the fights, for all the companionship.

Thomas, you have been so helpful to me many times, always kind and always ready to advert me to take care on work details that seem to be small, but that ended up being a great deal and help during my project. For this, and for your simple presence in the lab, danke!

陆怡, dear Lucy, we passed through all this two years together, and I’m so grateful for such great moments of compassion and solidarity that we shared with each other. All the fights we took to obtain this degree are now about to reach the end. Congratulations for the amazing work you have been developing and I wish you a lot of success in your promising future towards a colder country!

Christiane, during this year I felt that we were not two independent research groups, but one single unified group. Thank you for welcoming me ‘at home’, because from this I have experienced how it feels to belong to a friendly research group.

To all my dear lab mates in Stockholm, which promoted such a great work environment when I was so far from home, thank you so much for your hospitality and flexibility to give me some of your work time so that I could perform my measurements.

Special thanks to Jan and Joachim for opening the doors of their houses so that I could stay for a night in a more comfortable place.
Eva-Mari Aro, thank you for the seeds of the mutants I studied and all the knowledge that, without noticing, you transmitted to me through the many papers I have read over the past several months.

To my parents, Leonor e Zé, to whom I dedicate this work, not only for all this years of support regarding my professional education, but for all the education that they granted me which founded the roots of the human being I am today. Muito, muito, muito obrigado!

Queridos avós, tenho sempre presente a vossa imagem no meu dia-a-dia. Visualizar o vosso rosto dá-me força para continuar o caminho que faço longe de casa. Obrigado por suportarem a minha ausência. Apesar de longe, quero-vos perto, quero-vos todo o bem!

To my extended family, especially to my aunt Lena and my uncle João, that moved by curiosity and by being involved in the intricacies of this huge field that is Chemistry, always accompanied the developments of my work and supported me with means that pushed me forward. Obrigado 😊

Moi drodzy Weronika, Michał and Aleksandra, you have truly been my support along this year of performing this work, I’m truly grateful to you for every moment that we spent together, which was always a time to relief the stress of work. Dziękuję bardzo!

Last, but definitely not least, I cannot forget to mention os meus queridos amigos João e Sandra, which had been here for me since the day I met Umeå, three and a half years ago. To live and study in Umeå has a special and marvellous taste which I will never be able to dissociate from you.

I can’t say if choosing Umeå as a place to study and live for 2 years was an easy or a difficult choice, but I know it was a choice made with heart. For all those which I’m not able to mention on this section, but that through different ways contributed for the great experience of the past 2 years, I assure you that I keep a place for you in my heart.

Thank you all, for all your love and friendship! May the Light be with you!

Obrigado por todo o amor e amizade! Que a Luz esteja sempre convosco!

“Love is the one thing we're capable of perceiving that transcends dimensions of time and space. Maybe we should trust that, even if we can't understand it.”

- Interstellar, 2014
References


[17] D. A. Los and N. Murata, ‘Membrane fluidity and its roles in the perception of


Figure 1 – [56]; Figure 2 – [57]; Figure 3 – Adapted from [14]; Figure 4 – [58]; Figure 6 – Molecular dynamics visual investigation from the structure [59]; Figure 7 – [60]; Figures number 5, and 8-14, are creation of the author.
Appendix

Appendix 1 – Photography of experimental setups

Supplementary Figure 1 – Pictures of cultivated Arabidopsis thaliana, about 11 weeks of age, before the leaf harvesting for experiments: a, wild-type Columbia 0. b, STN7 mutant. c, STN8 mutant.

Supplementary Figure 2 – Example of Thin Layer Chromatography plates: to the left, a resolved TLC plate stained with a gentle flux of iodine to detect the two strongest bands corresponding to MGDG and DGDG; to the right, a TLC plate after scratching out the silica gel containing the desired lipids.
Appendix 2 – Data Analysis

Table 1 – Descriptive statistics of PAM data, $F_v/F_m$ measurements for the 3 observed strains: WT, STN7 and STN8.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>STN7</th>
<th>STN8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.789</td>
<td>0.80065</td>
<td>0.75755</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.002761</td>
<td>0.003089</td>
<td>0.003688</td>
</tr>
<tr>
<td>Median</td>
<td>0.79</td>
<td>0.8</td>
<td>0.761</td>
</tr>
<tr>
<td>Mode</td>
<td>0.79</td>
<td>0.8</td>
<td>0.761</td>
</tr>
<tr>
<td>Standard Deviation</td>
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<td>0.013816</td>
<td>0.016494</td>
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<tr>
<td>Sample Variance</td>
<td>0.000152</td>
<td>0.000191</td>
<td>0.000272</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>1.546548</td>
<td>0.523846</td>
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</tr>
<tr>
<td>Skewness</td>
<td>-0.86238</td>
<td>0.098946</td>
<td>-0.42071</td>
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<tr>
<td>Range</td>
<td>0.053</td>
<td>0.056</td>
<td>0.062</td>
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<tr>
<td>Minimum</td>
<td>0.756</td>
<td>0.772</td>
<td>0.725</td>
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<tr>
<td>Maximum</td>
<td>0.809</td>
<td>0.828</td>
<td>0.787</td>
</tr>
<tr>
<td>Sum</td>
<td>15.78</td>
<td>16.013</td>
<td>15.151</td>
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<tr>
<td>Count</td>
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<td>20</td>
<td>20</td>
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<tr>
<td>Confidence Level(95.0%)</td>
<td>0.005778</td>
<td>0.006466</td>
<td>0.007719</td>
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<table>
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<th>STN7 $F_v/F_m$</th>
<th>STN8 $F_v/F_m$</th>
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<tr>
<td>SD</td>
<td>0.789 ± 0.012</td>
<td>0.801 ± 0.014</td>
<td>0.758 ± 0.016</td>
</tr>
<tr>
<td>CI</td>
<td>0.789 ± 0.006</td>
<td>0.801 ± 0.006</td>
<td>0.758 ± 0.008</td>
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</table>

Table 2 – T-test for populations assuming equal variances: WT vs STN7 (left), WT vs STN8 right.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Mean</td>
<td>0.789</td>
<td>0.80065</td>
</tr>
<tr>
<td>Variance</td>
<td>0.000152</td>
<td>0.000191</td>
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<tr>
<td>Observations</td>
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<tr>
<td>Pooled Variance</td>
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<tr>
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</tr>
<tr>
<td>df</td>
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<td></td>
</tr>
<tr>
<td>t Stat</td>
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<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
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<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.685954</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.007748</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.024394</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>WT</th>
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<tr>
<td>Mean</td>
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<td>0.75755</td>
</tr>
<tr>
<td>Variance</td>
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<td>0.000272</td>
</tr>
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Table 3 - ANOVA test on $F_v/F_m$ measurements for the three populations: WT, STN7 and STN8

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<tr>
<th>Summary of Data</th>
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<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
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<tr>
<td>N</td>
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<td>20</td>
<td>20</td>
<td></td>
<td></td>
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<tr>
<td>$\Sigma X$</td>
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<td>15.151</td>
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<td>$\Sigma X^2$</td>
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<td>12.8244</td>
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<td>Std.Dev.</td>
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<td>0.0231</td>
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<table>
<thead>
<tr>
<th>Result Details</th>
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<tr>
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<td>df</td>
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<tr>
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<td>0.0099</td>
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<td></td>
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</tbody>
</table>

The $F$-ratio value is 48.46792. The $p$-value is < .00001. The result is significant at $p < .05$. 
Supplementary Figure 3 – Example of the outcome of a fitting session of an experiment. Multiple FAF were fitted for 10 different GUVs (DOPC 40% - DGDG 45% - MGDG 15%) with WT S1 integrated on the membrane. Curves were plotted with normalization to the same particle number. If not calculated, from the plotted graph one can visually estimate $\tau_D$, the diffusion time, by finding $\tau$ for $G(\tau)_{\text{max}} / 2$. 