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**Exploration of Nonphotochemical Quenching Mechanisms in *A. thaliana* via Time
Correlated Single Photon Counting Snapshots**

By

Michelle Layne Leuenberger

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Graham R. Fleming, Chair
Professor Ronald C. Cohen
Professor Krishna K. Niyogi

Summer 2017

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Abstract

Exploration of Nonphotochemical Quenching Mechanisms in *A. thaliana* via Time Correlated Single Photon Counting Snapshots

By

Michelle Layne Leuenberger

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University of California, Berkeley

Professor Graham R. Fleming, Chair

As described in chapter 1, during photosynthesis, plants harvest light energy from the sun and, through a series of several steps, convert it to chemical energy to be stored for later use driving cellular processes vital to life. However, under high light conditions, often more light energy is absorbed than can be used for productive photosynthesis. Because excess absorbed energy can cause severe damage to the photosynthetic reaction center proteins, it must be dissipated harmlessly as heat in order to protect the plant. In the first step of photosynthesis, light energy is absorbed by pigment protein complexes designed especially for light harvesting, called light harvesting, or antenna complexes. Because of their location relative to reaction centers, pigment composition, and their density, most absorbed light energy passes through antenna complexes before reaching reaction centers, making them ideal sites for photoprotective quenching, or nonphotochemical quenching. Nonphotochemical quenching, or NPQ, is the reduction in chlorophyll *a* fluorescence yield caused by the dissipation of excess excitation energy by mechanisms other than photochemistry. Under high light conditions, NPQ switches the function of light harvesting complexes to dissipate the energy they collect as heat in order to protect the reaction centers from damage when their capacity for productive photosynthesis is overwhelmed.

The induction of NPQ opens up a new relaxation pathway for electronically excited chlorophyll molecules by altering the distance of the excited chlorophyll from, and/or orientation relative to, a quencher. Neighboring chlorophylls and other xanthophyll pigments have been proposed as potential quenching molecules and as of yet, none have been ruled out and some experimental evidence exists to support each possible quencher. One way to change the distance between, and/or relative orientation of pigments within a pigment protein complex, or PPC, is a conformational change of the PPC. Previous work has demonstrated that the function of some integral biological membrane proteins can be modulated by the lipid composition in the membrane, which in turn modulates the lateral pressure profile, and thereby the protein conformation. Chapter 2 describes fluorescence lifetime measurements taken on LCHII embedded proteoliposomes with different lipid compositions. The results reveal increased quenching in the presence of the nonbilayer forming lipid MGDG, suggesting that the quenching is induced by an increase in lateral pressure in the acyl region of the membrane bilayer. LHCII is likely able to undergo a conformational change modulated by the lipid composition in the

thylakoid membrane, which brings relevant pigments closer to one another to allow for the harmless dissipation of excess energy in the form of heat.

In chapter 3, two xanthophyll cycles linked to NPQ, the violaxanthin cycle (VAZ cycle) and the lutein epoxide cycle (LxL cycle), are discussed. The cycling of xanthophylls affects the kinetics and extent of the photoprotective response triggered. While the VAZ cycle is ubiquitous among vascular plants and has been studied extensively, the LxL cycle is found in only about 60% of plants studied thus far and does not exist in model plants. Lauriebeth Leonelli, in the Niyogi lab, introduced the LxL cycle into *Arabidopsis thaliana* and functionally isolated it from the VAZ cycle. We showed an increase in dark-acclimated PSII efficiency associated with Lx accumulation. Time correlated single photon counting (TCSPC) measurements were performed to quantify the dependence of the response of NPQ to changes in light intensity on the presence and accumulation of zeaxanthin and lutein. Changes in the response of NPQ to light acclimation were observed between two successive light acclimation cycles, suggesting that xanthophyll cycles modulate the rapid component of NPQ necessary to prevent photoinhibition. Mathematical models of the response of zeaxanthin- and lutein-dependent reversible NPQ were constructed that describe the modulation. Finally, the wild-type response of NPQ was reconstructed from isolated components with a single common scaling factor, enabling deconvolution of the relative contributions of zeaxanthin- and lutein-dependent NPQ.

Chapter 4 describes TCSPC measurements at several excitation and detection wavelengths to determine the location of quenching in a new mutant of *Arabidopsis thaliana*. In 2013 the Niyogi lab characterized a new mutant, *soq1*, which displayed a novel form of qI quenching dependent on the protein, SOQ1. Further chemical mutagenesis on the *soq1* mutant revealed a second mutant, *soq1 otk1*, that displayed severe, constitutive quenching. Further characterization and TCSPC snapshot experiments taken at several excitation and detection wavelengths on the *soq1 otk1* mutant suggest that the constitutive quenching observed in *soq1 otk1* is likely occurring in LHCII trimers. The measured lifetimes are commensurate with lifetimes of aggregated LHCII trimers reported in the literature.

Lastly, in chapter 5, the data analysis methods developed to mitigate issues such as very large data sets, low counts, and error analysis are discussed. The MatLab code is provided in an appendix at the end of the chapter.

To my dearest father, Richard A. Leuenberger

August 14th, 1951 – March 14th 2012

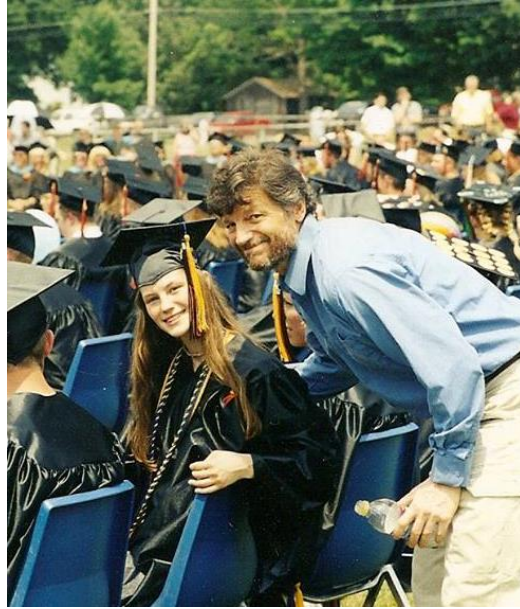


Table of Contents

Table of Contents	ii
Acknowledgements	v
Chapter 1: Introduction to Nonphotochemical Quenching, or NPQ, and Spectroscopic methods of studying NPQ	1
Optimization of Photosynthesis to Feed a Growing Global Population	1
Photosynthesis: A brief introduction as it pertains to NPQ	1
Nonphotochemical Quenching, or NPQ	5
Fluorescence Spectroscopy Sheds Light on Chlorophyll Excitation and Relaxation Dynamics	8
Fluorescence Yield measurements via Pulse Amplitude Modulated (PAM) Fluorimetry.....	8
Time Correlated Single Photon Counting, or TCSPC, and the TCSPC snapshot method.....	10
References	15
Chapter 2: Lipid composition in the thylakoid membrane modulates lateral pressure exerted on transmembrane proteins, likely thereby inducing conformational changes in LHCII trimers	19
Introduction: Theory of membrane lateral pressure impact on transmembrane proteins	19
Materials and Methods	22
Results	24
Discussion	27
Supplementary Information and Figures.....	28
References	30
Chapter 3: Dissecting and modeling zeaxanthin- and lutein-dependent nonphotochemical quenching in <i>Arabidopsis thaliana</i>	34
Introduction	34
Results and Discussion.....	35
Amplitude-Weighted Average Lifetimes via TCSPC	37
Modeling.....	39
Lutein-Dependent Quenching.....	43
Zeaxanthin-Dependent Quenching.....	46
Constructing wt Quenching from Components	47
Conclusion.....	50
Materials and Methods	50
Plant Material and Growth Conditions	50
TCPSC Measurements.....	51

Data Analysis.....	52
Monte Carlo Methods to Determine Available Xanthophyll Pools	52
Model Estimation	53
References	54
Chapter 4: Fluorescence lifetime studies on <i>Arabidopsis thaliana</i> mutants reveal a novel quenching mechanism localized to LHCII and modulated by a system comprised of the SOQ1 and OTK1 proteins, and a chloroplast lipocalin, AtCHL	58
Introduction	58
Materials and Methods	61
TCPSC Measurements.....	61
Data Analysis.....	62
Results	62
Discussion	66
Conclusion.....	67
Supplementary info/figures:.....	69
References:	70
Chapter 5: Development of Data Analysis Methods for TCSPC Snapshots	73
Introduction	73
Dealing with low photon counts and big data	78
Code Description.....	79
FitFramework.m	80
FitFluoLifetimeNoPlot.m	81
PlotFitTCSPCNoPlot.m.....	82
CalcError.m	82
FindRange.m.....	82
Convolve.m.....	83
InitialGuessFinder.m	83
DataPostProcess2comp.m.....	83
getstepsRCCv2.m	83
References	84
Chapter 6: Conclusions and Outlook	85
Conclusions	85

Outlook.....	85
Appendix A: MatLab code for data analysis procedure described in chapter 5	87
FitFramework.m	87
FitFluoLifetimeNoPlot.m	91
PlotFitTCSPCNoPlot.m	94
CalcError.m	95
FindRange.m	97
Convolve.m	99
InitialGuessFinder.m	100
DataPostProcess2comp.m	105
getstepsRCCv2.m	117

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When I arrived in Berkeley in August of 2012, I had just lost my father unexpectedly, and I struggled profoundly to survive my first years in the PhD program. Although it was incredibly difficult, there were many people who showed kindness and compassion while providing support in critical ways. I would like to first thank my advisor, Graham Fleming, for giving me the opportunity to be in his group and for having the energy and patience to help me pursue my own variation of success. Thank you, Graham, for the space and autonomy you gave me, for laughing at my jokes, and for recognizing that I needed somebody to tell me to worry less, rather than more. Thank you for your many insightful observations and bits of sound advice, and even for teaching me to pipe down and listen sometimes. Thank you, Graham, for hiring excellent female scientists and setting the example that the work of women in your lab will be respected and valued just as much as the work of their male counterparts. It wasn't the easiest ride, and there were times I was afraid I would not make it to the end, but the grand sum total is that Graham provided me with what I needed to succeed at every step of the way, and for that I cannot thank him enough.

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winning, while at the same time instilling in me the grace to enjoy the game even in the event of a loss. He would have loved to see my laser, to learn about my research, and to explore Berkeley – I cannot begin to express how much I miss him or wish I could have shared this experience with him. He would be so proud, and I imagine he would have said my laser was, “neat!”. I could not have asked for a better father or for more from the wonderful father I was so fortunate to have. He gave every bit of himself to my mother, sister, and to me with every breath he took – never missing an event or an opportunity to make us feel loved and protected. Thank you, Dad from the very bottom of my heart for every joke, every smile - for teaching me that mischievous smile, the one where you’re a little proud and very amused by your own joke. Losing a parent makes you realize just how instrumental parents are in creating the world we perceive around us. Thank you from the bottom of my heart, Dad, for the world you created for me and the life you gave me.

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After thanking them as individuals, I want to thank my parents as a unit. I want to thank them for being my biggest supporters, coming to every sporting event, dance recital, and play, and for showing me how to be the best person I could be. Together they modeled incredible strength, integrity, and perseverance. My mother, with her restless mind that flits from thought to thought in a kind of brilliant fireworks display, contrasted by my (relatively) quiet and contemplative father who took his time to solve problems systematically. Sometimes I feel the two of them battling within me - I hit a groove, get into a zone and I jump from paper to paper, jotting down racing thoughts, frenetically running through all of the possible explanations I can think of for my experimental results - racing along like my mother. Yet there are just as many occasions when I look around at the chaos and I see that the only way forward is to slow down and come

up with a step by step plan. When ‘the mom approach’ fails, the ‘dad approach’ is sure to save me. Together, they gave me the best of themselves. I love you both so much and could not have wished for better parents.

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And last but certainly not least, Berkeley - Berkeley, my heart. This is the place that embraced me, respected me, and even elevated me during the absolute most difficult years of my life thus far. Berkeley gave me space to fall down and pick myself back up again. I never imagined having this acceptance, this embrace - a community like I've never known but I've always wished for. For that, Berkeley, you will always have my heart.

Chapter 1: Introduction to Nonphotochemical Quenching, or NPQ, and Spectroscopic methods of studying NPQ

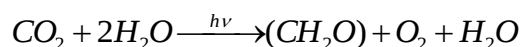
Optimization of Photosynthesis to Feed a Growing Global Population

Photosynthesis is crucial to life on earth as it is the source of all food and oxygen consumed by humans and other organisms, and it consumes carbon dioxide that would otherwise be harmful to life on earth. However, the United Nations Food and Agricultural Organization predicts the global population will exceed ten billion by the year 2050, and the global food supply will need to increase by 50% from 2013 levels in order to meet the dietary needs of that population [1]. Because meeting those needs via expansion of agricultural lands is not feasible due to high cost and limited availability of land, scientists believe the best approach to the food supply problem is to grow crops that produce higher yields. In a very recent and promising new development, researchers hypothesized that the yields of some major crops can be more than doubled when particular aspects of photosynthesis are optimized to increase biomass output [2].

In this chapter, I will briefly introduce photosynthesis and then delve into the process that is the subject of this thesis, nonphotochemical quenching, or NPQ. NPQ is one of the several processes involved in photosynthesis that has the potential to be further optimized in order to increase crop yields. The last section of this chapter will introduce the spectroscopic methods employed in the body of this thesis work to study NPQ in the hopes of providing further information that could facilitate improved biomass output of important crops.

Photosynthesis: A brief introduction as it pertains to NPQ

Photosynthesis is the process by which plants harvest light energy from the sun and, through a series of several steps, convert light energy to chemical energy to be stored for later use driving cellular processes vital to life [3]. Equation 1 is the net equation for (oxygenic) photosynthesis. In this section, a brief introduction of photosynthesis will be provided for the purpose of facilitating a better understanding of the need for NPQ, the machinery (proteins) involved in it, as well as the context and the physical environment in which the processes comprising NPQ take place. Only aspects vital to understanding NPQ will be addressed here. For a more complete treatment, please see references [3][4].



Equation 1

The photosynthetic apparatus is housed inside the thylakoid membrane, which is in turn housed in an organelle located in plant cells, called the chloroplast. Figure 1.1a shows a side-view of the inside of a leaf. The mesophyll cells, where most of the leaf photosynthesis occurs, are found beneath a wax cuticle and the upper epidermis, inside the cortex of the leaf. Inside the mesophyll cells, the chloroplasts house the photosynthetic machinery. Figure 1.1b shows a cartoon of the chloroplast and the thylakoid membrane within, and figure 1.1c shows the partitioning of the proteins comprising the photosynthetic apparatus within the thylakoid membranes: photosystem II, or PSII is mostly localized in the grana stacks along with the major light harvesting complex of PSII, light harvesting complex II, or LHCII. Photosystem I, or PSI, on the other hand is mostly found in the stroma lamellae along with ATP synthase. The major difference between these regions of the thylakoid membrane is that the stroma lamellae are

exposed to the stroma, or the fluid region that surrounds the thylakoid membrane, while the grana stacks are not. The grey background represents the lipid environment surrounding the proteins inside the thylakoid membrane. The entire thylakoid membrane is interconnected and encloses the space within, called the lumen.

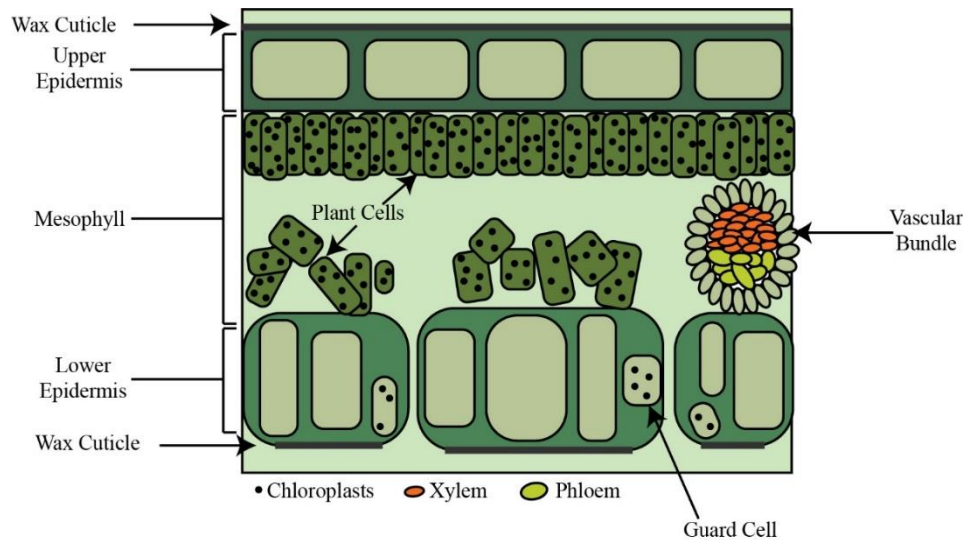


Figure 1.1a: the side-view of the inside of a leaf. The plant cells are found beneath a wax cuticle and the upper epidermis, inside the space of the mesophyll. Inside the plant cells, the chloroplasts, which house the photosynthetic machinery, are represented by black dots.

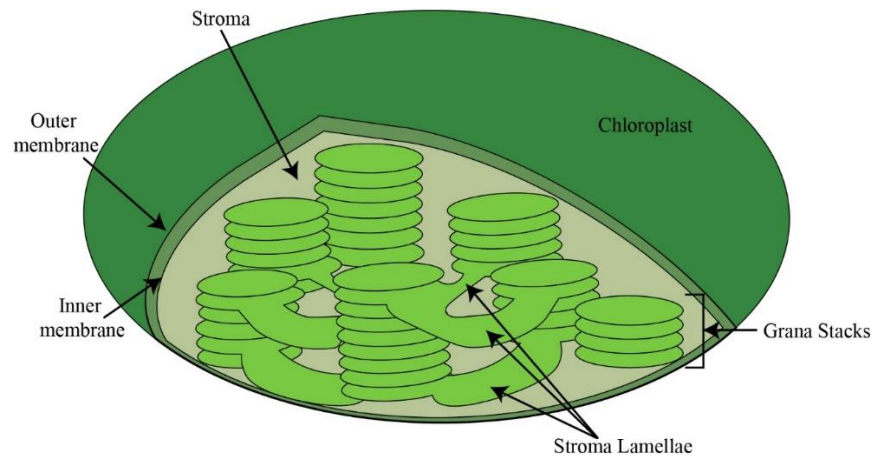


Figure 1.1b: a cartoon of the chloroplast and the thylakoid membrane within. The grana stacks are represented by discs stacked on top of one another while the stroma lamellae are the sections of membrane connecting the grana stacks.

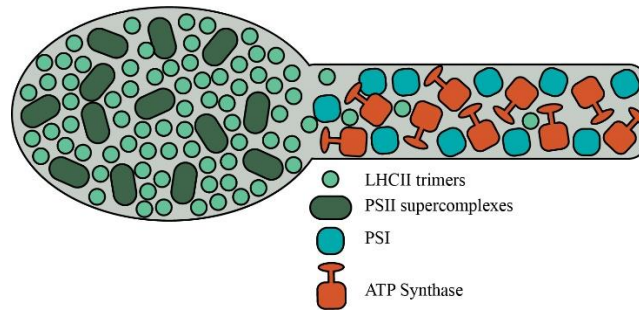


Figure 1.1c: The partitioning of the proteins of the photosynthetic apparatus within the thylakoid membranes: PSII is mostly localized in the grana stacks along with LHCII. PSI is mostly found in the stroma lamellae along with ATP synthase. The grey background represents the lipid environment that surrounds the proteins.

Taking a closer look, figure 1.2 is a schematic showing how the two photosystems work together. Note however, that in reality PSII and PSI are in different regions of the thylakoid membrane (except near the grana margins where the two regions meet). Although the representation is not physically accurate in the general sense, it is helpful to visualize the systems in this manner in order to understand the sequence of events that takes place during photosynthesis. There are two categories of reactions involved in photosynthesis, namely the light reactions and the carbon-fixing reactions. The light reactions produce molecular oxygen, ATP, and NADPH while the carbon-fixing reactions reduce carbon dioxide to carbohydrates and consume the ATP and NADPH generated by the light reactions. In short, the light reactions involving PSI, PSII, and their respective reaction centers, convert light energy to chemical energy. PSI and PSII are part of an electron transport chain which also includes cytochrome b_6f , a water-soluble copper protein called plastocyanin, and a lipid-soluble quinone, plastoquinone. As depicted in figure 1.2, the electron transport chain moves electrons from H_2O in the lumen, to soluble, redox active compounds in the stroma, such as $NADP^+$. Finally, ADP is phosphorylated on the surface of chloroplast ATP synthase, which along with NADPH produced by PSI, are the high energy products that drive the carbon-fixing reactions which take place in the stroma, the fluid surrounding the thylakoid membranes [4].

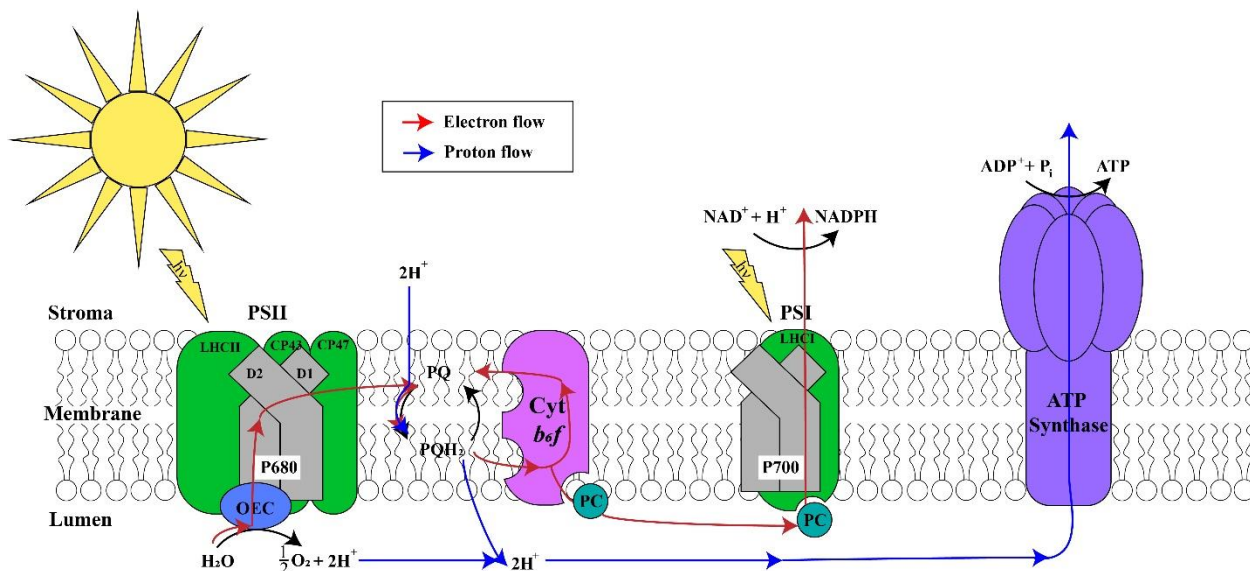


Figure 1.2: A schematic of the photosynthetic machinery inside the thylakoid membrane. Incident light, represented by the lightning bolt graphic, is absorbed by LHCII and leads to a charge separation in the PSII reaction center, P680. The subsequent path of electron and proton flow initiated by light absorption and the resulting charge separation are represented by red and blue arrowed lines respectively.

Under limited light conditions, each photon absorbed contributes to this process by causing a charge separation followed by subsequent, additional charge separation events which lead to the electron transport chain described above and eventually to the generation of ATP, NADPH, and then carbohydrates. However, under high light conditions when the rate of light absorption outpaces the plant's capacity for photosynthesis, the plastoquinones that maintain the charge separation by carrying electrons farther and farther from the PSII reaction center are overwhelmed and a bottleneck occurs. When the plastoquinone pool is fully reduced, the electron transport chain is halted and protons accumulate in the lumen establishing a pH gradient across the thylakoid membrane as the lumen becomes acidic while the stroma maintains a relatively neutral pH. This pH gradient, often referred to as ΔpH , acts as a trigger, initiating the processes which protect the photosynthetic machinery from damage caused by high light conditions. Although both of the photosynthetic reaction center complexes of higher plants, photosystem I (PSI) and photosystem II (PSII), experience photodamage and have photoprotective mechanisms, they are spectroscopically distinct, primarily because of the shallow nature of the PSII reaction center trap and the reversibility of primary charge separation in the PSII reaction center ($P680^+$) relative to PSI. These features lead to longer-lived excitation in the PSII reaction center and therefore, a higher probability of damage when reaction centers are closed. Moreover, the shallow trap and reversibility of charge transfer in PSII reaction centers contribute to variability in fluorescence from PSII, which allows for the study of nonphotochemical quenching (NPQ) via fluorescence yield and lifetime measurements [5]. The fluorescence from PSI is far less variable at room temperature. Furthermore, recent work suggests PSI has its own protective mechanism, likely dependent on cyclic electron flow and independent of NPQ entirely [6][7]. This thesis pertains to photoprotective mechanisms of PSII as observed via fluorescence spectroscopy.

Nonphotochemical Quenching, or NPQ

Nonphotochemical quenching, or NPQ, is the reduction in chlorophyll *a* fluorescence yield caused by the dissipation of excess excitation energy by mechanisms other than photochemistry [8]–[10]. These mechanisms comprise a set of processes plants employ to modulate the function of light harvesting complexes in order to adjust to fluctuations in light intensity seen by plants in nature, thereby ensuring protection under high light without compromising efficient photosynthesis under limited light. Although NPQ's various mechanisms allow for rapid response to excess light conditions, the overall response is slow to recover, leading to a period of potentially suboptimal photosynthetic efficiency [11]. Understanding the multiple processes underlying NPQ could inform engineering of photoprotective systems to increase crop yields [2][12].

The first step in the photosynthetic process involves absorption of light energy by pigment protein complexes designed especially for light harvesting, and aptly named light harvesting, or antenna complexes. These antenna complexes are arranged around the PSII reaction centers with subsets of antenna complexes weakly bound, moderately bound, strongly bound and even completely disconnected from the reaction centers as well as sometimes spatially segregated from reaction centers. Because of their arrangement around reaction centers, their pigment composition, and their quantity relative to other pigment protein complexes in the thylakoid membrane, most absorbed light energy passes through antenna complexes before reaching reaction centers, making them ideal protectors of reaction centers under saturating light conditions. When light is limited, the light harvesting complexes collect light energy and transfer it to the reaction centers of photosystem II, or PSII, with nearly 100% efficiency. In this case, 100% efficiency means that nearly every photon absorbed is used to drive photochemistry [3]. In contrast, under high light conditions, NPQ switches the function of light harvesting complexes to dissipate the energy they collect as heat in order to protect the reaction centers from lingering energy when their capacity for productive photosynthesis is overwhelmed. As can be seen in the Jablonski diagram depicted in figure 1.3, an excited chlorophyll molecule can transfer its energy to the S_1 electronic excited state of a neighboring chlorophyll. This relaxation pathway is the fastest, and therefore the most probable when light is limited. However, when light is plentiful, the plastoquinones which accept an electron from the PSII reaction center become fully reduced and can no longer accept electrons. In this case, charge recombination can occur, returning the chlorophyll to the excited electronic S_1 state. The relaxation pathway of energy transfer to neighboring chlorophylls, toward the reaction center is effectively closed under these circumstances and excitations live longer, thus increasing the probability of relaxation via one of the other depicted pathways, namely fluorescence, internal conversion, or intersystem crossing to a triplet state. Intersystem crossing to the triplet state is particularly dangerous because excited state triplet chlorophyll molecules can transfer energy to ground state (triplet) oxygen to produce excited singlet state oxygen and subsequently, other reactive oxygen species which can be very long-lived and cause extensive damage to the photosynthetic proteins.

Chlorophyll Excitation Manifold and Relaxation Pathways

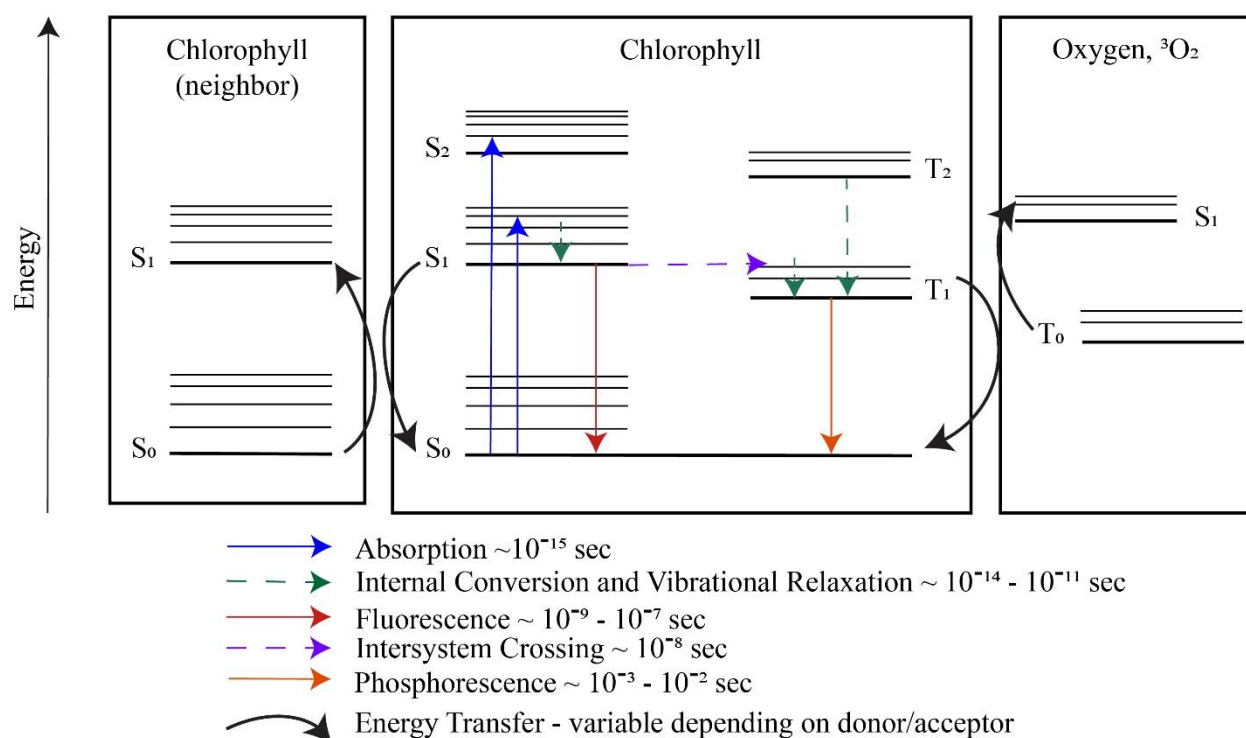
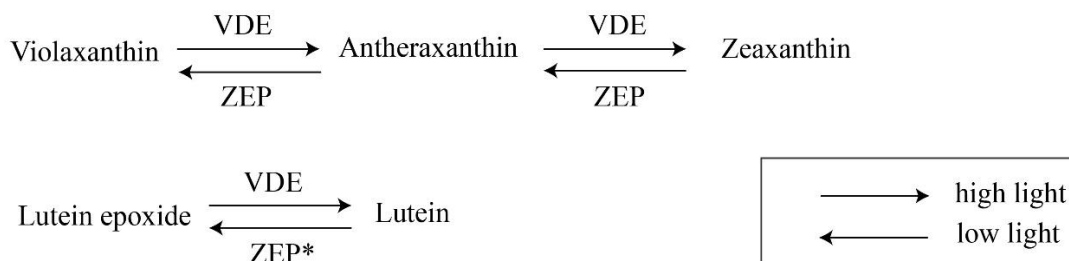


Figure 1.3: Chlorophyll excitation manifold and relaxation pathways. In the center of the diagram is a chlorophyll Jablonski diagram showing the excitation and relaxation pathways available to electronically excited chlorophyll molecules. The arrows depict different processes which are labeled beneath the figure along with their respective time scales. The first two electronic states of a neighboring chlorophyll are depicted in the left panel to show how energy can be transferred between neighboring chlorophyll molecules. The right panel depicts the ground triplet state, and the first excited singlet of molecular oxygen and the curved arrows represent energy transfer from excited state triplet chlorophyll molecules to ground state molecular oxygen, resulting in highly reactive singlet Oxygen.

As shown above in figure 1.2, the oxygen evolving complex of PSII, represented by a blue oval labeled, “OEC”, is positioned dangerously close to many excited chlorophyll molecules providing an endless supply of oxygen to receive energy from excited triplet state chlorophyll and thus, rendering NPQ a vital process to the survival of plants in nature. Consequently, plants have evolved to develop several protective mechanisms to prevent damage in PSII reaction centers. Carotenoids act as oxygen scavengers to stop damage, but are also more directly involved in quenching excited state chlorophyll before intersystem crossing can occur and thus before reactive oxygen species form in the first place, dissipating excess energy harmlessly as heat [14]. When the probability of relaxation via transfer of energy to a neighboring chlorophyll decreases (when reaction centers are effectively closed), the probability of relaxation via the remaining pathways increases, including fluorescence, which can be measured quantitatively and with picosecond time resolution in order to track the progression of NPQ induction and relaxation dynamics. Fluorescence spectroscopy is the most common method of studying NPQ and will be described in the next section of this chapter.

Although we understand the function of NPQ, the exact molecular mechanisms are not well understood. In fact, multiple such mechanisms have been suggested and it is not yet clear whether they are mutually exclusive. NPQ is a broad term encompassing several constituent

components historically categorized by rate of induction and relaxation. For the quenching response of PSII in vascular plants, the components are often separated into the rapidly reversible, energy-dependent quenching component (qE) and the slowly reversible component associated with PSII photoinhibition (qI) [15], [16]. Although important in many photosynthetic systems, a component of NPQ associated with excitation balance between PSI and PSII by altering the relative antenna size does not contribute significantly in vascular plants exposed to high light [8] and as such, will not be discussed in this thesis. The historic decoupling of rapidly reversible mechanisms from slower ones may not be as feasible as previously understood: subsequent work has indicated the complicated nature of the slow component called qI [17]–[21] and the roles of zeaxanthin in both a rapidly reversible and pH-dependent component called qE [22]–[27] and another component that depends on zeaxanthin but not pH termed qZ [28], making the distinction less clear and at times, arbitrary [9]. As of yet, there is little consensus surrounding the molecular mechanisms underlying the quenching pathways intrinsic to NPQ in PSII. However, several important players impacting the regulation of PSII photoprotection are widely agreed on. qE in higher plants is triggered by a high pH gradient (ΔpH) formed across the thylakoid membrane, because productive photochemistry resulting in charge separation outpaces the activity of ATP synthase and other downstream processes (see figure 1.3) [29]. PsbS, which contains exposed protonatable residues, has been shown to be a sensor of ΔpH [30] and is necessary for qE *in vivo* [31]. Finally, on the formation of ΔpH , violaxanthin deepoxidase (VDE) is activated, converting violaxanthin to antheraxanthin and zeaxanthin [32] in a cycle referred to by the abbreviation VAZ cycle. Some evidence suggests that zeaxanthin plays a direct role in quenching [24][25], whereas other evidence suggests that zeaxanthin simply regulates lutein-dependent quenching allosterically [33][34]. Although the accumulation of zeaxanthin from violaxanthin under high light conditions is ubiquitous among higher plants and has been studied extensively, an analogous cycle reflecting the accumulation of lutein from lutein epoxide (the LxL cycle), found in about 60% of plant species studied thus far, has recently become of interest [35]–[38]. Because this LxL cycle regulates lutein levels in response to light intensity changes in a similar way to the regulation of zeaxanthin in the VAZ cycle, the LxL cycle is of interest to help determine the impact of different xanthophyll cycles on the activation and recovery of NPQ in PSII. So far, the direct roles of zeaxanthin and lutein in NPQ as participants in either the molecular mechanism of quenching or the molecular regulation of quenching remain unclear. Both cycles will be addressed in this work and are shown in figure 1.4.



*ZEP in organisms lacking an LxL cycle is specific to Zeaxanthin and does not act on Lutein

Figure 1.4: The xanthophyll cycles relevant to NPQ of higher plants. VDE is violaxanthin de-epoxidase, and ZEP is zeaxanthin de-epoxidase.

Fluorescence Spectroscopy Sheds Light on Chlorophyll Excitation and Relaxation Dynamics

When chlorophyll molecules absorb light energy, they become electronically excited, meaning the electromagnetic wave induces an oscillating electric moment in the chlorophyll molecule which can lead to absorption of a photon if the frequency of the induced oscillation matches the frequency of light, and if the polarization of the light is not perpendicular to the transition moment itself. If the photon is absorbed, the molecule is said to be excited, and the distribution of charge on the molecule is less stable than it is in the ground electronic state. As depicted in Figure 1.3, the electronically excited chlorophyll molecule can relax back to the more electronically stable ground state by one of several pathways, occurring with different rates: 1. Energy transfer to a nearby chlorophyll molecule occurs in a matter of femtoseconds and is the fastest and most probable process, 2. Internal conversion and vibrational relaxation are also very fast and thus occur with relatively high probability, and 3. Intersystem crossing to an excited chlorophyll triplet state occurs with a rate on the order of nanoseconds as does 4. Fluorescence. As a result of the relative rates of the different relaxation pathways, when light absorption outpaces the capacity for photosynthesis in plants, energy transfer to neighboring chlorophylls no longer results in trapping at the reaction center and thus a larger fraction of excited chlorophyll molecules relax via the remaining pathways, including fluorescence that can be measured, and intersystem crossing which leads to damage. Under these circumstances, the lumen becomes acidified, a pH gradient is established, and a new relaxation pathway opens up which is faster than fluorescence and intersystem crossing and will therefore reduce the probability of relaxation via those pathways leading not only to a decrease in damage to the photosynthetic apparatus, but also to a measurable decrease in fluorescence that correlates with NPQ induction. Fluorescence spectroscopy is the most common method of studying NPQ and will be described in this section.

Fluorescence Yield measurements via Pulse Amplitude Modulated (PAM) Fluorimetry

As explained in the previous sections, chlorophyll fluorescence, and chlorophyll *a* fluorescence in particular in plants, has been used to study photosynthesis and NPQ non-invasively for decades. Plant biologists often use pulse amplitude modulated, or PAM fluorimetry to measure fluorescence yield in plants during dynamic light conditions. The experiment involves three different light sources with different purposes: a strong saturating pulse (up to 18,000 $\mu\text{mol photons/m}^2\text{s}$) used to close reaction centers, a weak measuring light ($< 0.1 \mu\text{mol photons/m}^2\text{s}$) intended to have minimal impact on photochemistry, and an intermediate intensity light source (0 – 3000 $\mu\text{mol photons/m}^2\text{s}$) used to adapt the sample to different light conditions mimicking sunlight of varying intensity. A diagram of a typical PAM trace on a wild type *Arabidopsis thaliana* plant is shown in figure 1.5 and the measured as well as calculated parameters from a PAM trace are found in table 1.1. During a PAM trace, a plant is first dark adapted for 30 minutes to ensure that the pigments involved in NPQ, mainly zeaxanthin, are in their inactive form at the start of the experiment. The measuring light is turned on and the baseline fluorescence yield, F_0 , is measured in the absence of a saturating pulse in order to determine the yield when reaction centers are still open. Next, a saturating pulse is applied to close the reaction centers and the fluorescence yield in dark with reaction centers closed, F_m , is measured. F_v , the maximum variable fluorescence, is the difference between F_m and F_0 . Next the actinic light is turned on and subsequent measurements are taken during light acclimation using the saturating pulse to ensure reaction centers are closed. These, and similar measurements taken once the

plant is returned to darkness to relax are referred to as F_m' . The NPQ parameter is calculated by taking the difference between F_m and F_m' and dividing it by F_m' , as shown in table 1.1. Additional useful measured, as well as calculated, parameters are listed in table 1.1. During each F_m' measurement, the plant is exposed to the saturating pulse for a total of 1 second, enough time to close reaction centers but not long enough to induce other process such as state transitions [39]. Although in dark adapted plants the time it takes to close reaction centers to saturation is fairly consistent, as NPQ turns on and off, it varies from snapshot to snapshot. To ensure that reaction centers are closed during these measurements, the one second measurement is broken into parts, which we typically refer to as steps, each 0.2 second in duration. Each step is analyzed separately and the step with the highest fluorescence yield is taken as the step with reaction centers closed. This background information and further details about PAM traces can be found here [40].

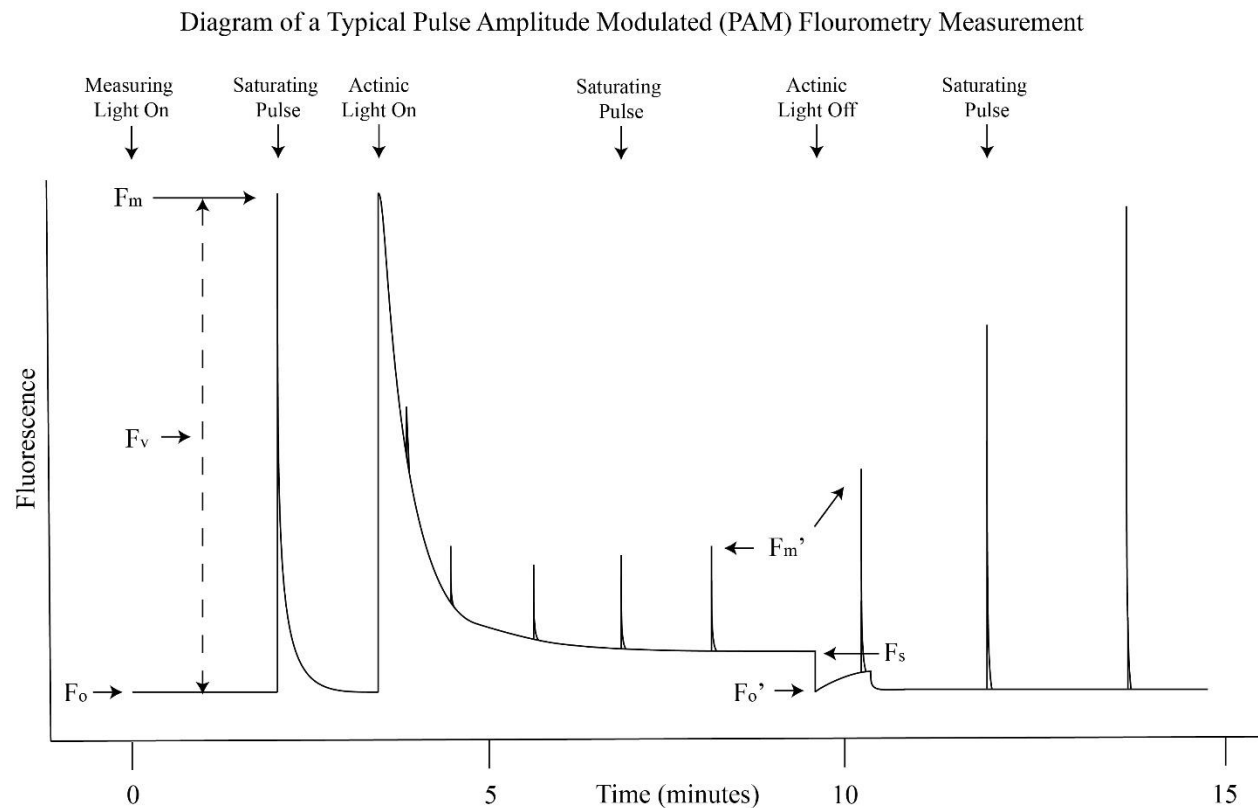


Figure 1.5: A diagram of a typical PAM trace of Col-0 wild type *Arabidopsis thaliana* leaves. See the table below for an explanation of the labeled features. The measuring light turns on at time zero and remains on throughout the experiment. Saturating pulses arrive periodically to close the reaction centers of PSII and measure the change in fluorescence yield as a result of light acclimation. The actinic light source, which mimics sunlight, is turned on after the first saturating pulse measurement. The purpose of the actinic light source is to induce nonphotochemical quenching.

PAM measured parameters			
F_o	Dark-adapted fluorescence yield. The fluorescence yield when all reaction centers are open	F_s	Fluorescence yield in light
F_m	Maximum fluorescence yield in dark, when all reaction centers are closed.	F_o'	Minimal fluorescence in a light-adapted leaf when all reaction centers are open
F_v	$F_m - F_o$	F_m'	Maximum fluorescence in a light-adapted leaf when all reaction centers are closed

PAM calculated parameters		
$\frac{F_v}{F_m}$	Maximum photochemical efficiency of PSII	
NPQ	$\frac{F_m - F_m'}{F_m'}$	NPQ of PSII fluorescence, directly proportional to energy dissipation
$\Phi PSII$	$\frac{F_m' - F_s}{F_m'}$	PSII operating efficiency

Table 1.1: An explanation of the labeled parameters in figure 1.5 is provided in the tables above.

The data obtained from PAM traces is quick, relatively easy to obtain, non-invasive, and as a result highly valuable, but it has its limitations. For example, fluorescence yield measurements are sensitive to chloroplast movement as well as photodamage to pigments, which can lower fluorescence yield but are unrelated to NPQ. Because average fluorescence lifetime is proportional to fluorescence yield, lifetime measurements provide the same information obtained from PAM traces, along with additional information. Moreover, fluorescence lifetime measurements are not sensitive to processes that decrease yield but leave decay times unaltered, such as chloroplast movement and photobleaching. In order to leverage the advantages of fluorescence lifetime measurements, Amarnath et. al. [31] and later, Sylak-Glassman et al. [42] in the Fleming lab, designed a setup to take fluorescence lifetime measurements during light acclimation in *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* respectively. The experimental design is modeled after that of a PAM trace and will be described in the following section.

Time Correlated Single Photon Counting, or TCSPC, and the TCSPC snapshot method

Time correlated single photon counting is the most common method of measuring fluorescence lifetimes. In TCSPC, a laser pulse excites the sample and, at a time t later, a fluorescence photon is detected and its time of arrival binned, with respect to a set of reference pulses. This is repeated many times until a histogram which is a convolution of the excited state fluorescence lifetime and the instrument response function, is built up. Similar to PAM traces, in TCSPC snapshot measurements the sample is exposed to the laser for a total of one second, and that period is then broken down into five steps, each 0.2 sec in duration. The fluorescence lifetime and the decay can then be extracted by fitting the data to a multi-exponential function using an exponential reconvolution method. The step with the longest lifetime, and thus with the reaction

centers closed, is retained. Figure 1.6 gives a visualization of this data acquisition structure. Figure 1.7 shows the averaged lifetimes measured during each step of a 1-second snapshot, taken on wt plants. Step three has the longest lifetime.

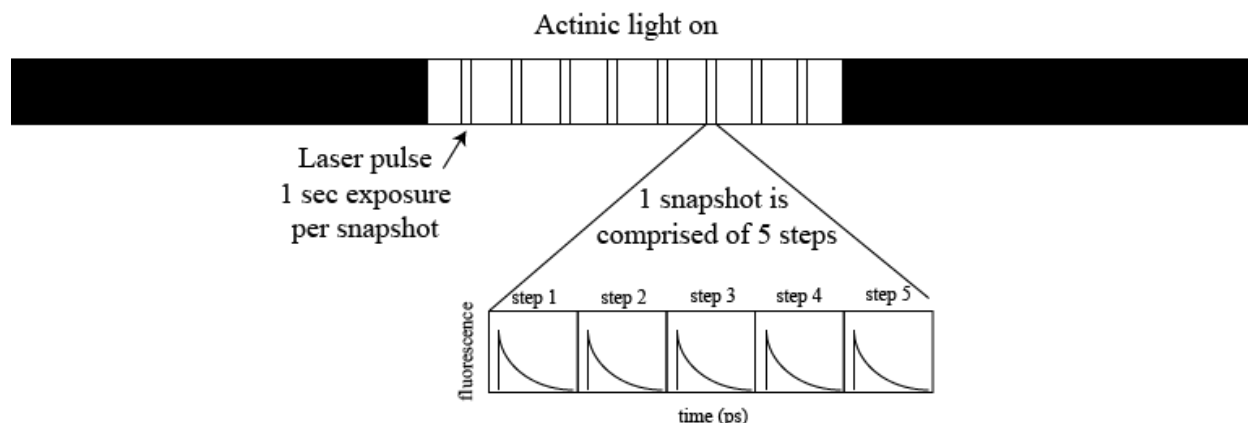


Figure 1.6: A schematic showing that each Snapshot consists of 5 measurements. The total snapshot time (the time of laser exposure) is 1 second. The snapshot is further broken down into 5 steps. The step with the longest average lifetime is the step with reaction centers closed to saturation.

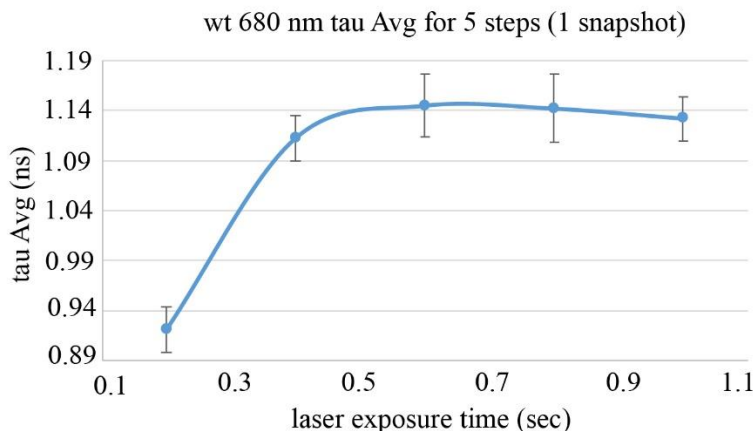


Figure 1.7: A plot of the average lifetimes obtained from the five steps comprising a snapshot taken on wt *Arabidopsis thaliana* leaves, excited at 420 nm and detected at 680 nm. The lifetime gets progressively longer initially as reaction centers are overwhelmed and closed to saturation, and then begins to decrease again. Step 3, the step with the longest lifetime is retained as the step with reaction centers closed to saturation.

The TCSPC snapshot setup employs a similar set of light sources with the exception that a laser beam acts as both the saturating pulse and the measuring light. The actinic light source is nearly identical to those used in PAM fluorimeters. As mentioned in the section above, one advantage of the fluorescence lifetime measurement over PAM fluorimetry, is that scenarios resulting in the same fluorescence yield, but different fluorescence lifetimes can be distinguished. Figure 1.8 shows several scenarios that could result in the same fluorescence yield (indistinguishable by PAM) but different fluorescence lifetimes (distinguishable via TCSPC). Panel 1.8a shows two decays: case A1, the solid line, illustrates quenching while case A2, the dashed line, illustrates bleaching. Bleaching reduces the number of fluorophores fluorescing but does not change the excited state lifetime of chlorophylls, whereas quenching does. Both scenarios may lead to

similar yields but they will likely lead to distinct lifetimes. TCSPC is advantageous because the measurements will not be influenced by bleaching or other processes that impact yield but not lifetime. Panel 1.8b illustrates another scenario in which the yields measured via PAM may be the same but the lifetimes may differ. In this case, B1 is a single exponential decay and is the likely result of weak quenching across all chlorophylls while B2, is a bi-exponential decay, indicating two chlorophyll populations with differing quenching capacities. Both curves may represent the same overall fluorescence yield, but their forms are clearly different and reveal distinct processes which are indistinguishable via PAM measurements.

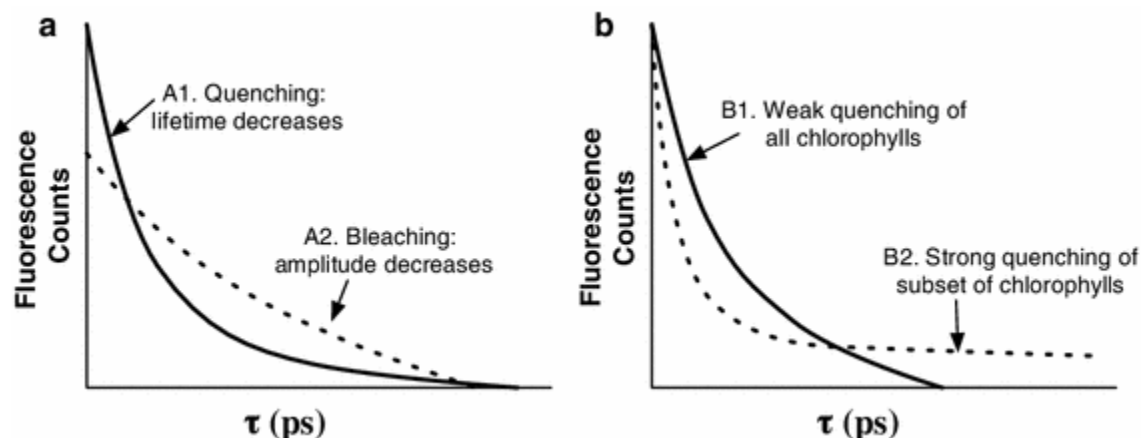


Figure 1.8: Scenarios giving rise to indistinguishable fluorescence yield measurements may be distinguishable by fluorescence lifetime measurements. a. Case A1, the solid line, illustrates quenching (decreased lifetime), while case A2, the dashed line, shows bleaching, marked by a decrease in amplitude. b. Fluorescence decays of moderate quenching of all fluorophores (case B1, solid line) and strong quenching of a subset of chlorophylls (case B2, dashed line).

Each fluorescence lifetime decay is analogous to an F_m' measurement in a PAM trace but it includes additional, time resolved information on a very short time scale. Whereas during a PAM trace, all photons detected during a period are simply summed, a TCSPC measurement bins the photons according to their arrival time in order to obtain picosecond time resolution, in addition to the minutes time resolution obtained from F_m' to F_m' measurement. As a result, a TCSPC experiment analogous to a PAM measurement is possible. Similar to PAM traces, plants are dark adapted for 30 minutes prior to the experiment and the first measurement is taken in dark to obtain the fluorescence lifetime in the relaxed state. Unlike a PAM trace which can be done on an entire plant, a leaf must be removed for the TCSPC experiment, and placed in a custom-built holder, pictured in figure 1.9, with a well to hold water for the petiole to take up, and an open clip, allowing the leaf to be exposed to air to avoid overheating. Then, an actinic light source is switched on to adapt the leaf and fluorescence lifetime measurements are taken at appropriate intervals during this time, until the light is turned off again, and additional measurements are taken to monitor NPQ relaxation dynamics. Figure 1.10 below is a cartoon of the experimental design.

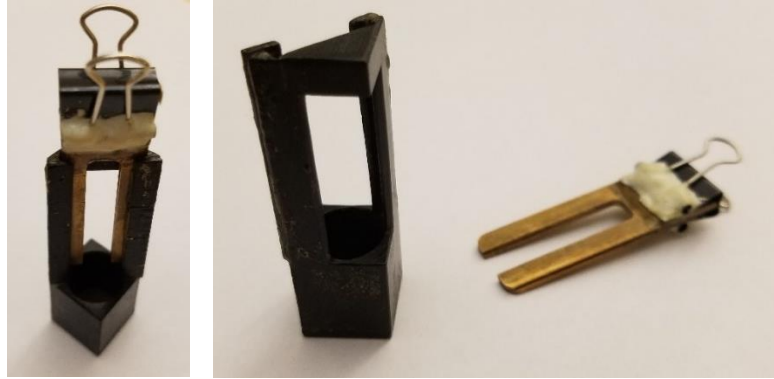


Figure 1.9: Home built sample holder for whole leaves of *Arabidopsis thaliana*. The sample holder has a built in well to hold 2 milliliters of water and the clip is constructed such that the majority of the leaf's surface, front and back, is exposed to air to avoid overheating and drying.

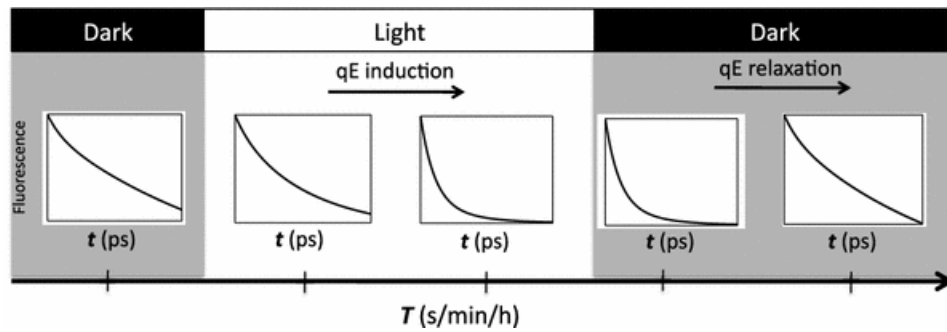


Figure 1.10: A cartoon showing the general concept of TCSPC snapshots. TCSPC is used to measure fluorescence lifetime decays in dark adapted leaves as they are acclimated to different lifetimes in order to elucidate further details of the NPQ mechanism.

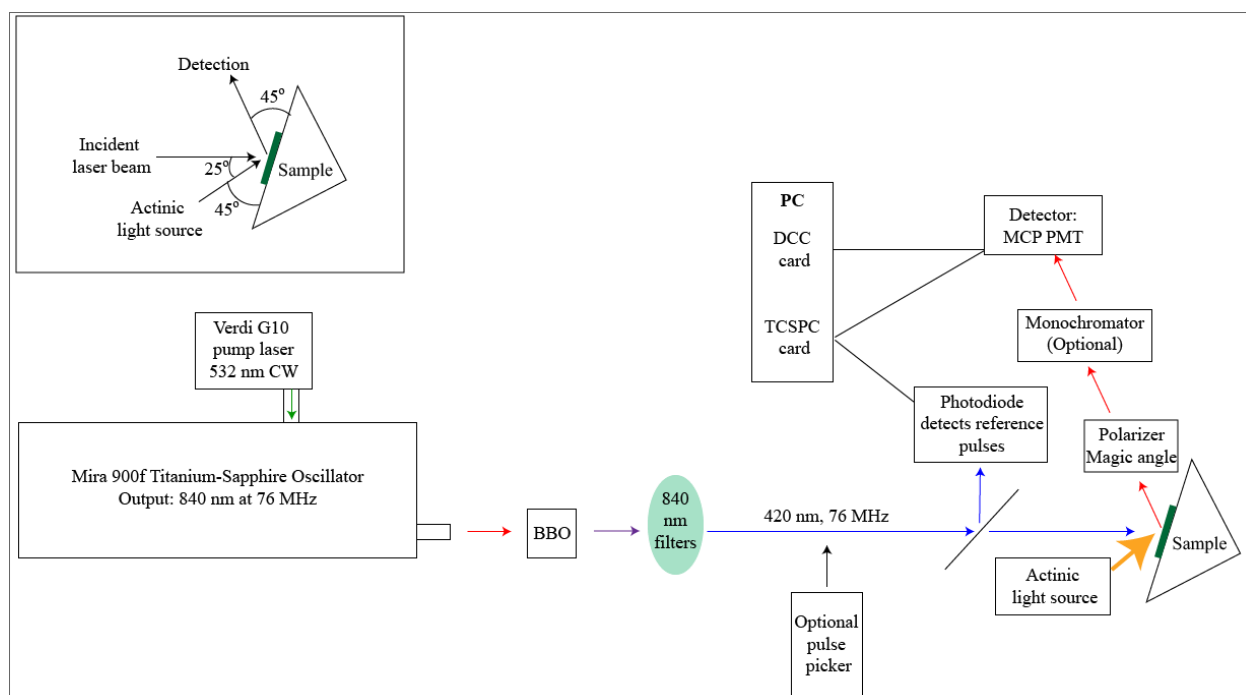


Figure 1.11: A map of the home-built TCSPC setup used in the Fleming lab and throughout this thesis. A Verdi G10 pump laser is used to pump the Mira 900f Oscillator with continuous wave 532 nm light, producing 840 nm light at a repetition rate of 76MHz. The 840 nm light is then frequency doubled using a Barium Borate Oxalate crystal to obtain 420 nm light at the same repetition rate. A pulse-picker can then be used to alter the repetition rate if necessary. A beam splitter sends a small fraction of the beam to a reference diode to detect reference pulses while the rest is incident on the sample at 70° angle to the adaxial side of the leaf. The fluorescence emitted from the leaf is then collimated and passed through a polarizer set to the magic angle, and then through a monochromator (or filter) to a microchannel plate photomultiplier tube detector. The detector and the reference diode relay the data to a Becker & Hickl TCSPC data acquisition card.

Figure 1.11 shows a diagram of the home-built TCSPC setup. A Coherent Verdi G10 pump laser is used to pump the Coherent Mira 900f Oscillator with continuous wave 532 nm light, producing 840 nm light at a repetition rate of 76MHz. The 840 nm light is then frequency doubled using a Barium Borate Oxalate, or BBO crystal to obtain 420 nm light at the same repetition rate. A pulse-picker can then be used to alter the repetition rate if necessary. A beam splitter sends a small fraction of the beam to a reference diode to detect reference pulses while the rest is incident on the sample at a 70° angle to the adaxial side of the leaf. The fluorescence emitted from the leaf is then collimated and passed through a polarizer set to the magic angle, and then through a monochromator (or filter) to a microchannel plate photomultiplier tube detector (Hamamatsu R3809U). The detector and the reference diode relay the data to a Becker & Hickl TCSPC data acquisition card. In this thesis, several experiments employing variations of this experimental design to study various sample types are described along with the insights the work has provided in the field of NPQ.

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Chapter 2: Lipid composition in the thylakoid membrane modulates lateral pressure exerted on transmembrane proteins, likely thereby inducing conformational changes in LHCII trimers

Collaborations: This work was done in collaboration with Stephanie Tietz in the lab of Helmut Kirchhoff at Washington State University. Samples were prepared and characterized by Stephanie Tietz and Emily Stafford in the Kirchhoff lab. A manuscript featuring the data described here in addition to single molecule experiments done by Bart van Oort is in progress in collaboration with Helmut Kirchhoff.

Introduction: Theory of membrane lateral pressure impact on transmembrane proteins

As described in chapter 1, the photosynthetic apparatus of vascular plants is housed in the thylakoid membrane, which is comprised of proteins densely packed into a lipid bilayer. According to the shape-structure concept of lipid polymorphism, described by Cullis et. al. in 1979, lipid preference for a particular structure upon self-assembly can be rationalized by the overall shape of the individual lipids as determined by the relative cross sections of the headgroup and the acyl chain [1]. In addition to chemical structure, environmental parameters such as pH (charged headgroup effectively larger), salt concentration (can neutralize charged headgroups), temperature, and the presence of divalent cations can alter the effective lipid shape and thereby its preferred structure of assembly in a given environment [1][2], offering a possible mode of modulating lipid structure *in vivo*. The shape-structure concept of lipid polymorphism divides lipids into several categories: type I lipids have a bigger head and smaller tail cross section and preferentially form aggregate structures with positive curvature, e.g. micelles, while cylindrical lipids are cylindrical in shape (head and tail similar in size) and tend to form a lipid crystalline lamellar phase similar to biological membrane bilayers, and so-called type II lipids form an aggregate structure with negative curvature preferred, such as the inverted hexagonal phase (H_{II}). See Figure 2.1 for a visual representation of these lipid categories and their preferred structures upon self-assembly.

Cullis and coworkers noticed that many biological membranes contain significant proportions of non-bilayer (type II) lipids even though the dominant structure in these environments is the lipid crystalline lamellar phase commonly associated with the naturally occurring lipid bilayers that make up biological membranes. They hypothesized that the pronounced presence of nonbilayer lipids inside biological membranes indicates a special role for these lipids. For example, although they are typically thought of as nonbilayer forming lipids, type II lipids make up about 50% of the lipids comprising the thylakoid membrane of vascular plants [3]. Another example is the *E. coli* inner membrane, which contains 75% nonbilayer lipid PE (phosphatidylethanolamine) [4]. Moreover, the balance of bilayer and nonbilayer lipids is highly regulated and vital to cell/organism viability, providing additional evidence of an important role for nonbilayer lipids in biological membranes [5] [6].

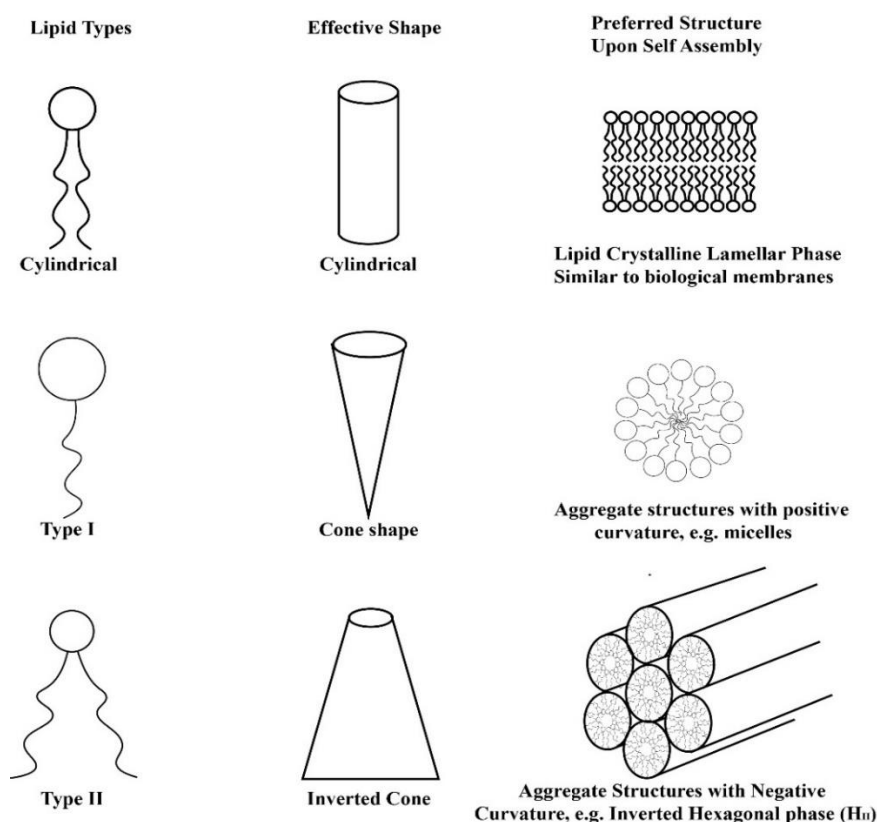


Figure 2.1: A cartoon drawing of three lipid categories and their preferred structures upon self-assembly.

Several studies have shown that lipid composition (in particular the abundance of nonbilayer lipids) can serve to modulate the lateral pressure profile of biological membranes, which in turn offers a mechanism of protein conformational change in cases where the change in conformation is accompanied by a depth-dependent variation in the cross-sectional area of the protein [2], [6]–[9]. As shown in Figure 2.2, lateral pressure p in a planar bilayer membrane changes along the membrane depth z and depends on the classification of the lipids comprising the membrane. Repulsions among hydrocarbon chains are stronger when nonbilayer forming lipids are present, causing the pressure in the hydrophobic acyl region of the membrane bilayer to increase, which can induce protein conformational changes that impact protein function [2]. Cantor et. al. provide a simple thermodynamic analysis which predicts the shift in the protein conformation equilibrium as a result of changes in the pressure profile [7]. Using this method, they demonstrated the high sensitivity of the lateral pressure profile within biological membranes to small changes in membrane composition and suggested that the resulting tunability of the pressure profile may serve to modulate proteins whose function involves a conformational change accompanied by a depth-dependent variation in the cross-sectional area of the protein [7]. Experimental evidence demonstrates modulation of the function of certain model proteins by membrane lipid composition as well. For example, the peripheral membrane protein Leader Peptidase $\Delta 2-75$ was shown to insert into phospholipid monolayers as well as vesicles with a preference for non-bilayer lipids [10], [11]. Moreover, increased membrane binding was observed for all nonbilayer lipids tested, suggesting that no specific interactions are involved, leading the authors to conclude that nonbilayer lipids create insertion sites due to a reduced packing density at the membrane water surface [11]. Studies on another model protein, KcsA,

demonstrated that alcohol-induced dissociation of the protein accompanied by a transition from an hourglass-shaped structure toward a more cylindrical-shaped structure is inhibited in the presence of nonbilayer lipids, suggesting that nonbilayer lipids stabilize the hourglass-shaped structure [12]. These two proteins are considered model proteins for a large class of peripheral and integral membrane proteins and as such, van den Brink-van der Laan et al. suggest that the observed effects of nonbilayer lipids reflect a general principle: nonbilayer lipids stimulate membrane binding of peripheral membrane proteins and affect the stability of (oligomeric) complexes of integral proteins via changes in the lateral pressure profile and thereby modulate the function of such proteins [12]. Perhaps more relevant to the proteins of interest inside the photosynthetic apparatus of vascular plants, it was also shown that the thermodynamic driving force for the meta I - meta II conformational change of the light responsive ocular pigment rhodopsin is tightly controlled by mixtures of nonbilayer forming lipids [13].

In light of these observations as well as *in vitro* studies by Bart Van Oort et. al. demonstrating pressure modulation of LHCII conformational quenching in the absence of aggregation [14], it was hypothesized that LHCII quenching *in vivo* could be modulated by thylakoid membrane lipid composition through the modulation of the lateral pressure profile of the membrane. LHCII photo-protective quenching involves changes in the connectivity of pigments involved in the energy transfer network of LHCII trimers in such a way that some antennae gain a photophysical pathway or mechanism with a rate of relaxation to the ground state that is fast relative to fluorescence and intersystem crossing (ISC). The energetic connectivity of pigments is determined by their orientation, separation from other pigments and their local protein environment. As described in chapter 1, the main players known to be essential for qE are ΔpH [15], which triggers changes leading to qE [16]–[21], the antenna complexes; LHCII [22]–[24], CP26 [25] and CP29 [26] are proposed to be the site(s) of quenching, the protein PsbS [27]–[29], which acts as a catalyst [30] and is activated by ΔpH [21], and Zeaxanthin [31], [32], which may work in conjunction with PsbS [30]. Some studies suggest Zea cannot function as an active quencher without PsbS [33] while others suggest that PsbS merely catalyzes the mechanism involving Zea [30]. Many hypotheses for qE mechanisms exist, most of which are not mutually exclusive, including excitonic coupling between Zea and Chl, leading to dissipation of energy via the Zea S_1 state [33], [34], formation of a Chl-Zea charge transfer state capable of quenching chl fluorescence [35], [36], and LHCII aggregation/LHCII conformational change which leads to quenching via energy transfer to the lut S_1 state by chl612 [37] and/or by a chl-chl charge transfer state that facilitates quenching [38]. Because energetic connectivity of pigments is determined by their orientation, separation from other pigments and local protein environment, this work focuses on mechanisms of quenching involving LHCII conformational changes. Based on the literature described above, we hypothesize that LHCII conformational changes may be induced by changes in the lateral membrane pressure profile modulated by the thylakoid membrane lipid composition. See figure 2.2 for further details.

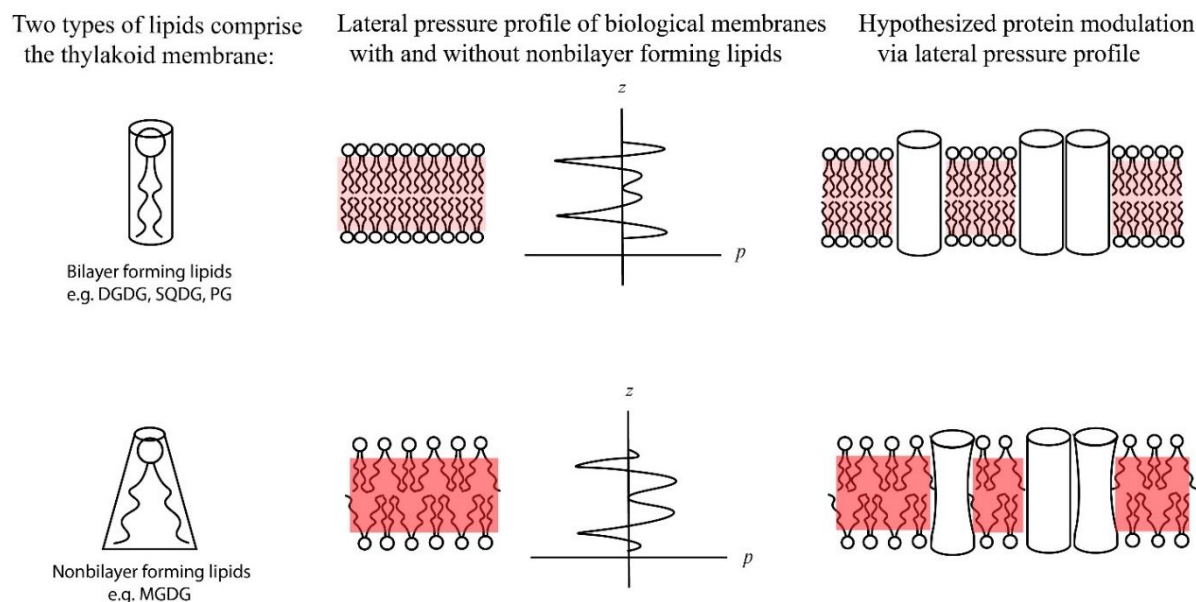


Figure 2.2: The thylakoid membrane is comprised of two types of lipids; bilayer forming such as DGDG, SQDG, and PG, and nonbilayer forming, namely MGDG. When nonbilayer forming lipids are incorporated into biological membranes, simulations suggest the lateral pressure profile is altered such that the pressure in the headgroup region is decreased while the pressure in the tail region of the membrane is increased significantly [39]. We hypothesize that the increased lateral pressure in the tail region of the membrane due to the presence of MGDG nonbilayer forming lipids induces a conformational change in LHCII trimers, thereby inducing a portion of NPQ related to LHCII conformational quenching. Figure modified from Kirchhoff et al. with permission.

The 4 main lipids comprising the thylakoid membranes of higher plants are glycerolipids, or fatty acids attached to a glycerol backbone via ester bonds. SQDG (Sulfoquinovosyl diacylglycerol) and PG (Phosphatidylglycerol) are bilayer lipids with negative charged headgroups, while DGDG (digalactosyldiacylglycerol) is a bilayer lipid with a non-ionic headgroup, and MGDG (Monogalactosyldiacylglycerol) is a nonbilayer lipid with a non-ionic headgroup [40]. We hypothesized that non-bilayer lipids (MGDG) can switch LHCII conformation by altering the lateral pressure profile in the hydrophobic core of thylakoid membrane. In order to test this, we conducted TCSPC fluorescence lifetime measurements on LHCII embedded proteoliposomes in order to confirm quenched fluorescence in the presence of the nonbilayer forming lipid MGDG, which makes up approximately 50% of the lipid composition in the thylakoid membrane in plants [3].

Materials and Methods

LHCII embedded proteoliposomes were prepared and characterized via 77K absorption spectra in the lab of Helmut Kirchhoff by Stefanie Tietz and Emily Stafford in order to show minimal free chlorophylls and minimal protein aggregation in the samples. LHCII trimers were isolated from spinach according to the procedure outlined in [41] and subsequently embedded in proteoliposomes with and without nonbilayer forming lipids according to the procedure in [42] but using a high-pressure extruder for liposome preparation [43]. See Figure 2.3 for the detailed procedure and Table 2.1 for sample compositions. The nonbilayer forming lipid used was MGDG, which makes up approximately 50% of the lipid composition in thylakoid membranes of vascular plants [3]. Fluorescence lifetime measurements were performed to determine whether

the presence of nonbilayer forming lipids, and the resulting increased pressure in the hydrophobic acyl part of the membrane bilayer, induce a conformational change in LHCII trimers that leads to chl *a* fluorescence quenching. LHCII trimer concentration was kept low relative to lipid composition (>11,000 lipids per LHCII trimer, compared to 70% protein are in native thylakoid membranes) in order to avoid aggregation and thereby isolate quenching effects due to the changes in lateral pressure profile from aggregation quenching, which is observed in the absence of pressure effects. Also, the concentration of MGDG in the proteoliposomes is only about 25% whereas it's closer to 50% *in vivo*. The reason for this is to avoid formation of the H_{II} inverted hexagonal phase in the proteoliposomes, which may contribute to other physiological effects including LHCII aggregation [44]. The samples are devoid of PsbS and Zea so it can be concluded that any quenching observed is unrelated to qZ or qE and can be attributed to conformational changes in LHCII induced by changes in lateral pressure most likely due to lipid composition.

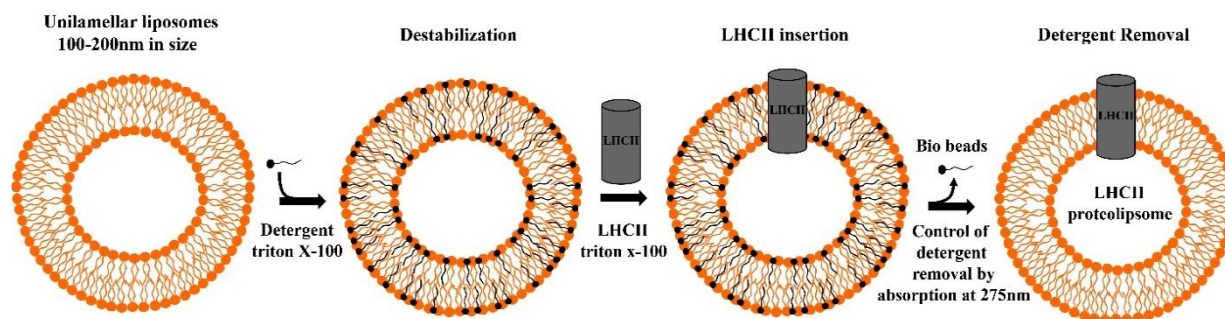


Figure 2.3: Shows procedure for proteoliposome sample preparation. Figure modified from Stephanie Tietz and Helmut Kirchhoff with permission.

Lipids/chlorophylls = 150 6,300 lipids per trimeric LHCII		
	Type I (+MGDG)	Type II (-MGDG)
MGDG	25 +/- 5%	4 +/- 1%
DGDG	46 +/- 5%	65 +/- 6%
PG	16 +/- 3%	18 +/- 4%
SQDG	13 +/- 3%	14 +/- 3%

Table 2.1: Lipid composition of sample types I and II, +MGDG and -MGDG respectively. The errors given as percentages are standard deviations. Samples were prepared and characterized in the lab of Helmut Kirchhoff by Stephanie Tietz. Permission was granted to use the data in this thesis.

Time correlated single photon counting (TCSPC) measurements of fluorescence lifetime were acquired using Becker-Hickl module SPC-150 in conjunction with Becker-Hickl SPCM software. The 420 nm excitation pulses with a repetition rate of 3.8MHz were generated by a Coherent Verdi G10 532nm diode pump laser which pumps a Coherent Mira 900f Ti:Sapphire

Oscillator set to 840nm. The resultant pulsed beam was then frequency doubled using a β -barium borate (BBO) crystal to obtain 420nm pulses at a repetition rate of 76 MHz. A pulse picker composed of a Harris SiO₂ crystal, and a Coherent 7200 cavity dumper in combination with an ENI voltage amplifier (model 403LA) to drive the acoustic waves was used to reduce the repetition rate to the desired 3.8MHz for these experiments. Samples were prepared to have an optical density of approximately 0.1. The sample cell used was a Starna cell with Spectrosil far UV Quartz windows, usable range: 170 – 2700nm, a 1mm path-length and a 0.4mL volume. In order to obtain sufficient fluorescence counts from the sample, a long pass filter was used to detect wavelengths longer than 650nm, instead of a monochromator. 420nm light was used to excite the chlorophyll *a* solet band. Fluorescence was detected using a Hamamatsu R3809U microchannel plate photomultiplier tube (MCP PMT) and the IRF had a full width half max of approximately 60ps. Measurements were taken at several powers and it was determined that there was no power dependence for measurements taken at average powers less than 100uW. Therefore, all measurements taken at powers below 100uW were averaged to obtain a larger sample size. Room temperature absorption spectra were taken using a Varian Cary 50 UV-vis spectrophotometer before and after each measurement to ensure the sample was not altered by the measurement. The data were fitted to bi-exponential functions via NLLS methods and reconvolution with the instrument response function using Picoquant Fluofit software, allowing for analysis of the average lifetime as well as the decay components and their amplitudes.

Results

Figure 2.4 shows the raw data for each measurement, as well as the averaged data across all measurements taken at powers less than 100uW. Both the raw and averaged data clearly show LHCII trimers are consistently, significantly more quenched when embedded in membranes containing nonbilayer MGDG lipids compared to those embedded in membranes containing only bilayer forming lipids.

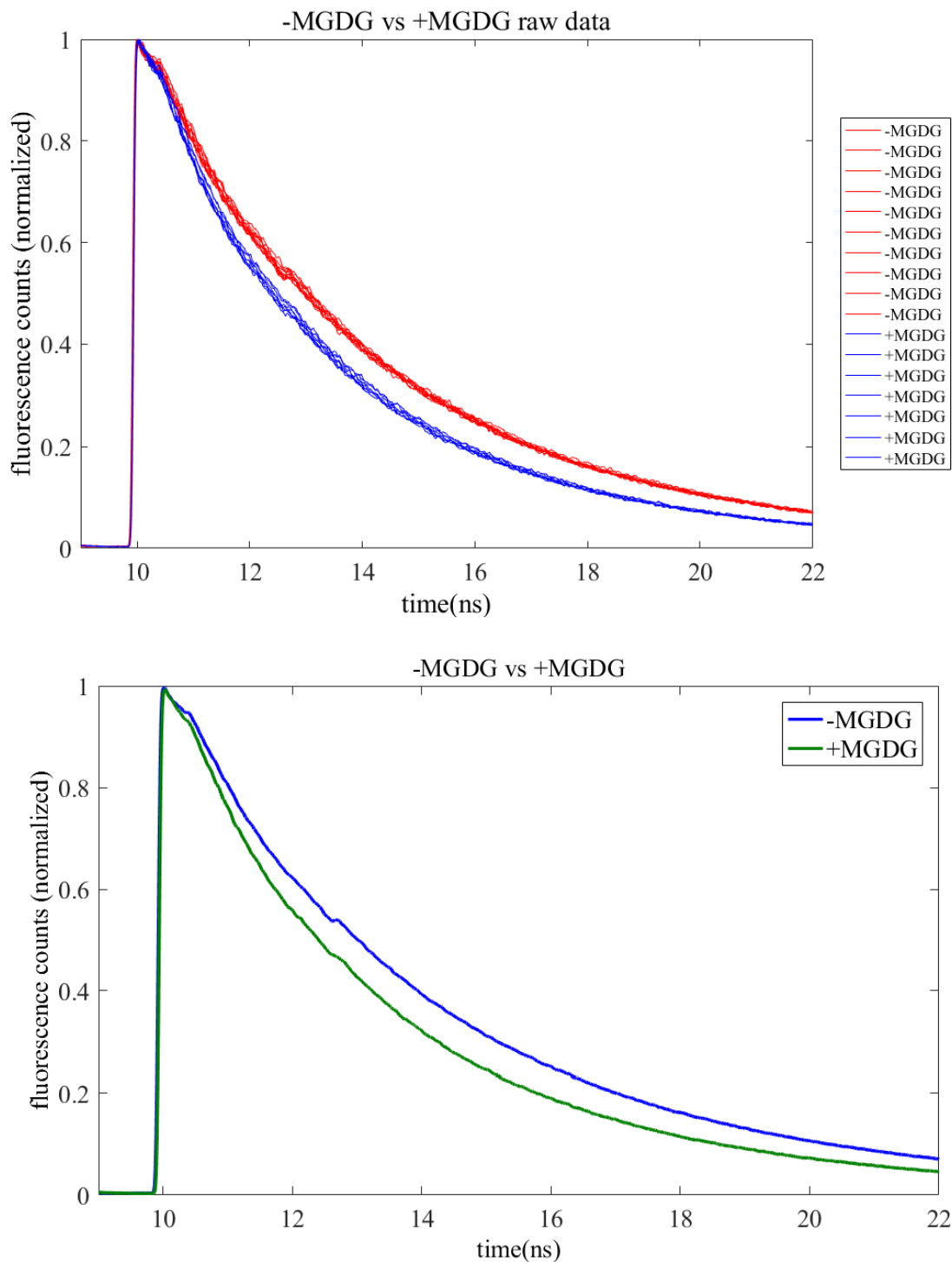


Figure 2.4: Raw fluorescence lifetime data for -MGDG (blue) and +MGDG (red) samples (A), and averaged data for -MGDG(blue) and +MGDG(green) samples. The averaged data clearly shows the +MGDG LHCII proteoliposomes display significantly reduced fluorescence lifetimes while the raw data indicates that the results are consistently replicated across several measurements and all measurements exhibit the trend seen in the averaged data: LHCII embedded in +MGDG proteoliposomes have a shorter decay time than those embedded in -MGDG proteoliposomes.

In addition, analysis of the lifetime components and their amplitudes (Table 2.2) is in agreement with previous work done by van Oort et. al. [14], suggesting that LHCII trimers can be stimulated to undergo quenching due to a conformational change induced by an increased hydrostatic pressure in the hydrophobic part of the lipid bilayer. The work done by van Oort et. al. differed from the experiments described here in that the LHCII trimers were floating freely in a buffer solution and experienced a relatively constant increase in pressure from all angles rather than experiencing lateral pressure changes as we would expect *in vivo*. In the van Oort et. al. work, the fluorescence lifetime of LHCII trimers was measured at various pressures and it was shown that the average fluorescence lifetime of the trimers did in fact decrease as pressure was increased in agreement with our results in proteoliposomes. Van Oort et al. also fitted their data to bi-exponential functions, resolving lifetimes similar to those measured here: a shorter component of approximately 0.5ns, and a longer component of approximately 3.5ns. These decay times differ slightly from ours as would be expected due to the different environment the samples are in. However, the values are similar enough to suggest that they may originate from the same processes. Furthermore, in the work of van Oort et al., the shorter decay time was shown to increase with increasing pressure, while the longer decay time decreased with increasing pressure. Figure 2.5 and table 2.2 show the two decay times as well as the average lifetimes associated with -MGDG and +MGDG samples and the same trends observed by van Oort et. al. can be seen here. In +MGDG samples, which we presume to have a higher pressure in the hydrophobic part of the lipid membrane bilayer, the shorter decay time is longer and the longer decay time is shorter than in the -MGDG samples. Lastly, van Oort et. al. saw a decrease in the ratio of the amplitude of the longer lifetime component with respect to that of the shorter lifetime component and the same trend was observed here. While -MGDG samples had an A_2/A_1 ratio of $0.848/0.152 = 5.58$, the +MGDG samples had an A_2/A_1 ratio of $0.814/0.186 = 4.38$.

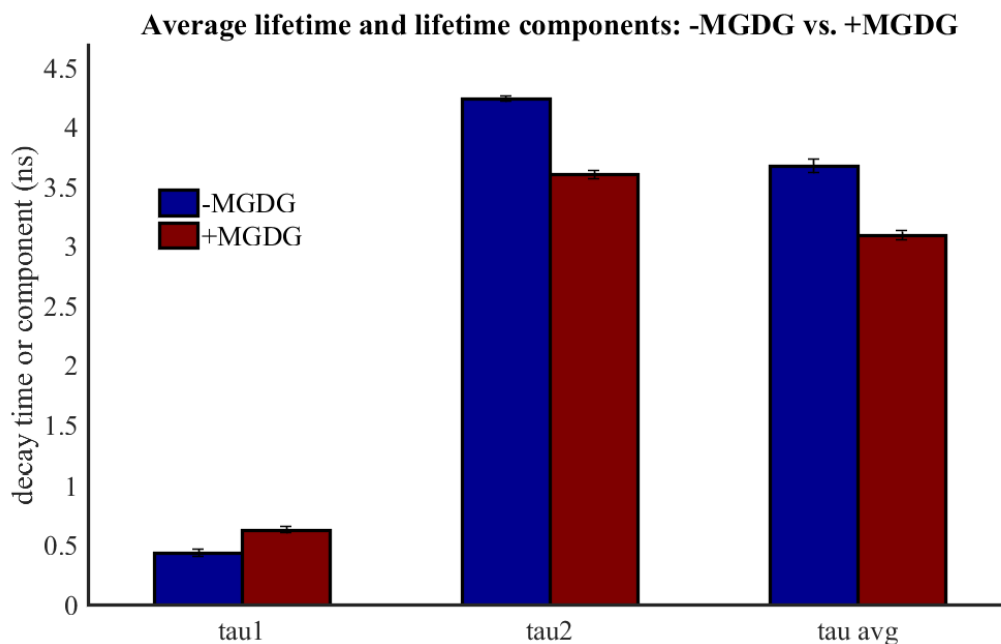


Figure 2.5: The decay times and average lifetimes measured for samples with (red) and without (blue) the nonbilayer forming lipid, MGDG.

	+MGDG		-MGDG	
	Average Value	Standard Deviation	Average Value	Standard Deviation
A_1	0.19	0.02	0.15	0.01
τ_1	0.63 ns	0.03	0.46 ns	0.04
A_2	0.81	0.02	0.85	0.01
τ_2	3.60 ns	0.03	4.20 ns	0.04
τ_{avg}	3.09 ns	0.06	3.67 ns	0.04

Table 2.2: Experimental results averaged across sample sets for +MGDG and -MGDG samples. The decay times are given in ns, while the amplitudes are presented as fractions. The errors are listed here as one standard deviation.

Discussion

Because no zeaxanthin or PsbS were present in the proteoliposome samples measured in this work, any quenching observed is most likely due to conformational changes in the embedded

LHCII trimers of +MGDG proteoliposomes relative to those embedded in -MGDG samples. Since the only differences between the samples was the nonbilayer:bilayer forming lipid composition, it seems probable that the quenching is induced by an increase in lateral pressure in the acyl region of the membrane bilayer. Based on previous work showing similar behavior of model proteins in response to changes in lateral pressure within the membrane in response to lipid structure modulation, this work supports the conclusion that LHCII is able to undergo a conformational change modulated by the lipid composition in the thylakoid membrane, likely accompanied by a change in the diameter of the trimer in the depths of the membrane which brings relevant pigments closer to one another to allow for the harmless dissipation of excess energy in the form of heat. While the observed effect is small relative to the full NPQ response and could only account for a portion of the typical NPQ response, recall that the concentration of MGDG was lower in the proteoliposomes than it is in thylakoid membranes of plants (25% in proteoliposomes vs. 50% in thylakoid membranes). At higher concentrations of MGDG, like those seen in thylakoid membranes, the impact could be stronger and account for a larger portion of observed NPQ.

Supplementary Information and Figures

A two-sample paired t-test was performed to validate the conclusion that the average lifetime of +MGDG samples is significantly shorter than that of -MGDG samples. Because the two sample sizes are different, in order to do the test some data points had to be ignored. In order to ensure that the results are a true representation of whether the average values are different, the eliminated data points were chosen to decrease the difference between the averages. The data assessed is found in table S2.1. The highlighted values were eliminated to make the sample sizes comparable. Elimination of these values should not strongly impact the difference in averages and would only reduce the difference, ensuring that the t-test will return a true determination of whether the averages are the same or different.

T-test for tau avg	
MGDG+	MGDG-
3.006817599	3.677403149
2.997279066	3.638135248
3.117460283	3.628944606
3.035666568	3.629281051
2.988181376	3.686250137
3.136602872	3.660374681
3.050823122	3.63055109
	3.650308271
	3.671903574
	3.745556178

Table S2.1: Data used for two sample paired t-test. The highlighted values at the bottom of the -MGDG column were eliminated in order to obtain similar sample sizes.

The null hypothesis was chosen to be that the two averages are the same. The results of the T-test can be found in table S2.2 below. The p values are on the order of 10^{-7} , far smaller than the chosen 0.05 threshold, and the t stat value of -22 is far less than the t critical values, both of

which indicate that the averaged average lifetimes of +MGDG samples are significantly shorter than those of -MGDG samples.

t-Test: Paired Two Sample for Means		
	<i>Variable 1</i>	<i>Variable 2</i>
Mean	3.047547269	3.65013428
Variance	0.003446787	0.000594025
Observations	7	7
Pearson Correlation	-0.353786682	
Hypothesized Mean Difference	0	
df	6	
t Stat	-22.42756643	
P(T<=t) one-tail	2.57075E-07	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	5.14151E-07	
t Critical two-tail	2.446911851	

Table S2.2: Table of t-test results. The p values are far less than the chosen threshold of 0.5 and the t stat is far less than t critical values, both of which indicate that the averaged average lifetimes of +MGDG samples are significantly shorter than thos of -MGDG samples.

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Chapter 3: Dissecting and modeling zeaxanthin- and lutein-dependent nonphotochemical quenching in *Arabidopsis thaliana*

Published work: This work has been published in PNAS [1] and was co-authored with Jonathan Morris, Arnold Chan, Lauriebeth Leonelli, Krishna Niyogi, and Graham Fleming. Jonathon Morris contributed equally to authorship. Michelle Leuenberger obtained and analysed the data while Jonathan Morris developed the model.

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Introduction

Photosynthesis begins with solar-driven electron transfer in reaction centers [2], but often, the energy available from sunlight outpaces the capacity for productive photochemistry in photosynthetic organisms. This mismatch can cause serious damage to the proteins that make up the photosynthetic apparatus. The fluctuations in light intensity experienced by higher plants necessitate both the rapid induction of photoprotective processes in response to high light conditions to prevent photodamage and subsequent relaxation of quenching to ensure optimal photosynthetic activity on return to low light conditions [3][4]. Although both of the photosynthetic reaction center complexes of higher plants, photosystem I (PSI) and photosystem II (PSII), experience photodamage and have photoprotective mechanisms, they are spectroscopically distinct, primarily because of the shallow nature of the PSII reaction center trap and the reversibility of primary charge separation in the PSII reaction center (P_{680}^{+}) relative to PSI. These features lead to longer-lived excitation in the PSII reaction center and therefore, a higher probability of damage when reaction centers are closed. Moreover, the shallow trap and reversibility of charge transfer in PSII reaction centers contribute to variability in fluorescence from PSII, which allows for the study of nonphotochemical quenching (NPQ) via fluorescence yield and lifetime measurements [5]. The fluorescence from PSI is far less variable at room temperature.

Here, we discuss photoprotective mechanisms of PSII as observed via fluorescence lifetime measurements. The suite of photoprotective mechanisms that protect PSII collectively results in and is referred to as NPQ: the reduction in chlorophyll *a* (Chla) fluorescence yield caused by the dissipation of excess excitation by mechanisms other than photochemistry [6]–[8]. Although NPQ’s various mechanisms allow for rapid response to excess light conditions, the overall response is slow to recover, leading to a period of potentially suboptimal photosynthetic efficiency [9]. Understanding the multiple processes underlying NPQ could inform engineering of photoprotective systems to increase crop yields [4] or systems to protect bioinspired energy devices [10].

NPQ is a broad term encompassing several constituent components historically categorized by rate of induction and relaxation. For the quenching response of PSII in vascular plants, the components are often separated into the rapidly reversible, energy-dependent quenching component (qE) and the slowly reversible component associated with PSII photoinhibition (qI) [11][12]. Although important in many photosynthetic systems, a component of NPQ associated with excitation balance between PSI and PSII by altering the relative antenna size does not contribute significantly

in vascular plants exposed to high light [6]. The historic decoupling of rapidly reversible mechanisms from slower ones may not be as feasible as previously understood: subsequent work has indicated the complicated nature of the slow component called qI [13]–[17] and the roles of zeaxanthin in both a rapidly reversible and pH-dependent component called qE [18]–[23] and another component that depends on zeaxanthin but not pH termed qZ [24], making the distinction less clear and at times, arbitrary [8].

As of yet, there is little consensus surrounding the molecular mechanisms underlying the quenching pathways intrinsic to NPQ in PSII. However, several important players impacting the regulation of PSII photoprotection are widely agreed on. qE in higher plants is triggered by a high pH gradient (Δ pH) formed across the thylakoid membrane, because productive photochemistry resulting in charge separation outpaces the activity of ATP synthase and other downstream processes [25]. PsbS, which contains exposed protonatable residues, has been shown to be a sensor of Δ pH [26] and is necessary for qE in vivo [27]. Finally, on the formation of Δ pH, violaxanthin deepoxidase (VDE) is activated, converting violaxanthin to antheraxanthin and zeaxanthin [28] in a cycle referred to by the abbreviation VAZ cycle. Some evidence suggests that zeaxanthin plays a direct role in quenching [20], [21] whereas other evidence suggests that zeaxanthin simply regulates lutein-dependent quenching allosterically [29], [30]. So far, the direct roles of zeaxanthin and lutein in NPQ as participants in either the molecular mechanism of quenching or the molecular regulation of quenching remain unclear.

Although the accumulation of zeaxanthin from violaxanthin under high light conditions is ubiquitous among higher plants and has been studied extensively, an analogous cycle reflecting the accumulation of lutein from lutein epoxide (the LxL cycle), found in about 60% of plant species studied thus far, has recently become of interest [31]–[34]. Because this LxL cycle regulates lutein levels in response to light intensity changes in a similar way to the regulation of zeaxanthin in the VAZ cycle, the LxL cycle is of interest to help determine the impact of different xanthophyll cycles on the activation and recovery of NPQ in PSII. Recently, transgenic lines of *Arabidopsis thaliana* modified to express zeaxanthin epoxidase from the alga *Nannochloropsis oceanica* have been produced [35], allowing the study of the isolated LxL cycle in a well-characterized model system. In this paper, we present spectroscopic studies of these lines and other xanthophyll mutants, and we examine the contribution of zeaxanthin- and lutein-dependent activation of NPQ in PSII to the full WT response.

Results and Discussion

To distinguish lutein-dependent quenching from zeaxanthin-dependent quenching, chlorophyll fluorescence lifetime snapshot measurements of whole leaves of *A. thaliana* sensitive to changes in Chl a fluorescence in PSII were collected via time-correlated single-photon counting (TCSPC). The genetic lines studied include WT (*wt*; Columbia-0 ecotype), the *szl1* mutant that lacks VAZ cycle xanthophylls and accumulates high levels of lutein [36], the double mutant *szl1npq1* that is also defective in VDE activity [36], the lutein-deficient mutant *lut2* [37], the novel lutein epoxide cycle transgenic mutant *szl1+NoZEP1* [35], and lastly, the transgenic mutant *szl1npq1+NoZEP1*, which constitutively accumulates lutein epoxide and lacks zeaxanthin and therefore, cannot induce any rapidly reversible qE [35]. The mutants studied and their properties are supplied in [Table 3.1](#).

Name	Mutation	Effect
Col-0 (wt)	N/A	N/A
<i>lut2</i>	Mutated lycopene ϵ -cyclase (LCYE)	Mutated LCYE inhibits formation of α -carotene and lutein.
<i>szl1</i>	Mutated lycopene β -cyclase (LCYB)	Mutated LCYB partially inhibits β -carotene formation and severely restricts accumulation of zeaxanthin, antheraxanthin, and violaxanthin.
<i>szl1npq1</i>	Mutated lycopene β -cyclase (LCYB) and mutated violaxanthin de-epoxidase (VDE)	Mutated LCYB partially inhibits β -carotene formation and severely restricts accumulation of zeaxanthin, antheraxanthin, and violaxanthin. Mutated VDE inhibits formation of zeaxanthin from antheraxanthin and violaxanthin.
<i>szl1+NoZEP1</i>	Mutated lycopene β -cyclase (LCYB), addition of zeaxanthin epoxidase from <i>Nannochloropsis oceanica</i> (NoZEP1)	Mutated LCYB partially inhibits β -carotene formation and severely restricts accumulation of zeaxanthin, antheraxanthin, and violaxanthin. Lutein is converted to lutein epoxide in dark conditions by NoZEP1, and lutein epoxide is converted to lutein in high light conditions by VDE, forming the LxL cycle.
<i>szl1npq1+NoZEP1</i>	Mutated lycopene β -cyclase (LCYB) and mutated violaxanthin de-epoxidase (VDE), addition of zeaxanthin epoxidase from <i>Nannochloropsis oceanica</i> (NoZEP1)	Mutated LCYB partially inhibits β -carotene formation and severely restricts accumulation of zeaxanthin, antheraxanthin, and violaxanthin. Mutated VDE inhibits formation of zeaxanthin from antheraxanthin and violaxanthin and lutein from lutein epoxide in high light conditions. Lutein is converted to lutein epoxide in dark conditions by NoZEP1.

Table 3.1: Lines of *A. thaliana* studied with explanation of mutations and effect on xanthophyll content and dynamics

After 30 min of initial dark acclimation, TCSPC snapshots were collected during two periods of high light, each followed by a period of dark relaxation. The first cycle consisted of 20 min of high light, which is long enough to achieve quasisteady-state quenching lifetimes, followed by 10 min of darkness, chosen to be long enough to allow a return to steady-state unquenched lifetimes but short enough to maintain high levels of deepoxidized xanthophylls in plants with appropriate epoxidase and deepoxidase enzymatic cycles. The second cycle, which immediately followed the first, consisted of 7 min of high light followed by 3 min of darkness and served to show the response of the fluorescence lifetimes when the deepoxidized carotenoids are present at the onset of the induction of rapidly reversible qE. Additionally, the pigments present in leaf samples exposed to the same light cycles were quantified via high-performance liquid chromatography (HPLC). [Fig. 3.1](#) summarizes these data.

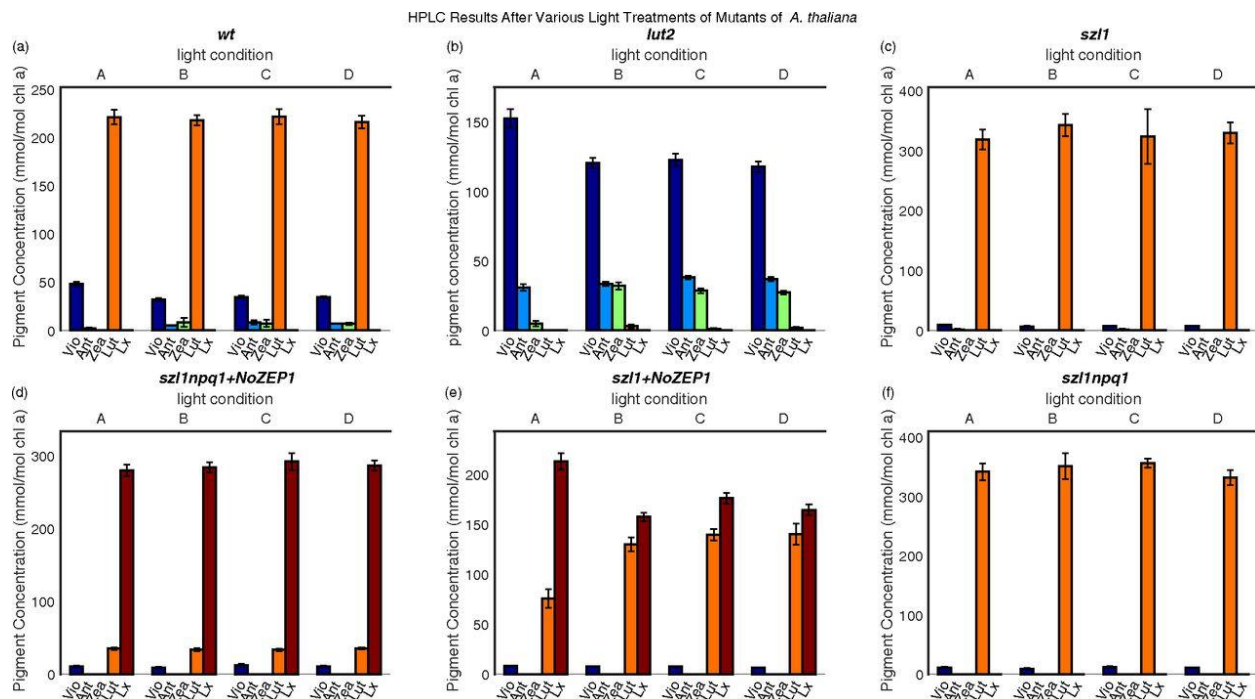


Figure 3.1: HPLC results for mutants of *Arabidopsis thaliana* after light treatment corresponding to TCSPC snapshot measurements. (A–F) Light conditions for sets A–D consist of initial dark acclimation (set A) followed by a scheme corresponding to the light acclimation scheme used for TCSPC measurement (in the text). Set B corresponds to samples after the first 20 min of light acclimation of the scheme. Set C corresponds to samples after the next 10 min of dark acclimation. Set D corresponds to samples acclimated to the subsequent light–dark cycle of 7 min of high light followed by 5 min of dark.

Amplitude-Weighted Average Lifetimes via TCSPC

Representative fluorescence decays measured via TCSPC in the quenched and relaxed states of *wt* leaves are provided in Fig. 3.2. For the two decays shown, the dark-acclimated or relaxed average lifetime was 1.42 ns, and the quenched average lifetime obtained after 270 s of 745 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ actinic light acclimation was 0.52 ns. The average lifetimes were obtained from an amplitude-weighted average of the fit of data to two lifetime components. The decision to fit the data to two lifetime components was made on analysis of a singular value decomposition (SVD) of the dataset (Fig. 3.3) that resulted in two singular vectors containing structure and remaining singular vectors displaying noise. The SVD result indicates that fitting more than two components does not extract additional meaningful information, reflecting the tradeoff between limited data collection time of snapshot measurements and the dynamic range of the fluorescence decay curves.

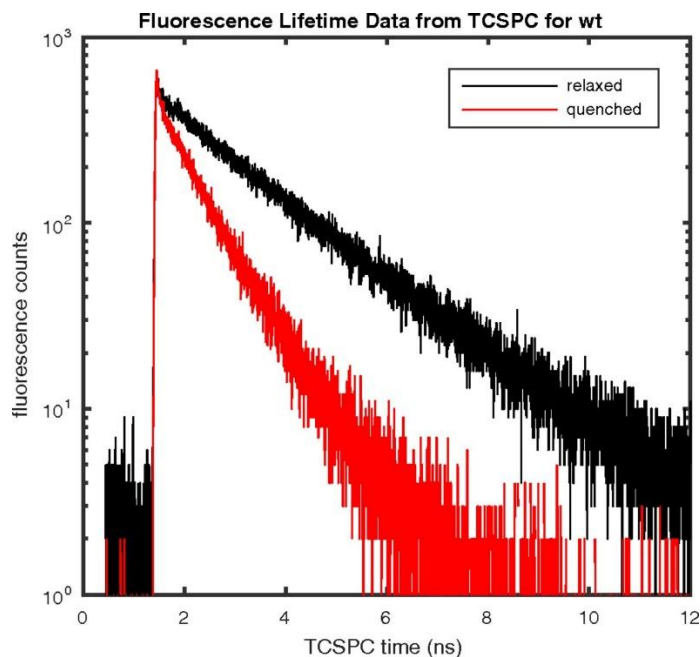


Figure 3.2: Representative fluorescence lifetime decays for dark-acclimated (relaxed) and light-acclimated (quenched) WT *A. thaliana* leaves. The average lifetime obtained for the sample shown was 1.42 ns for the relaxed state and 0.52 ns for the quenched state after 270 s of exposure to $745 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ actinic light.

Singular Value Decomposition Results

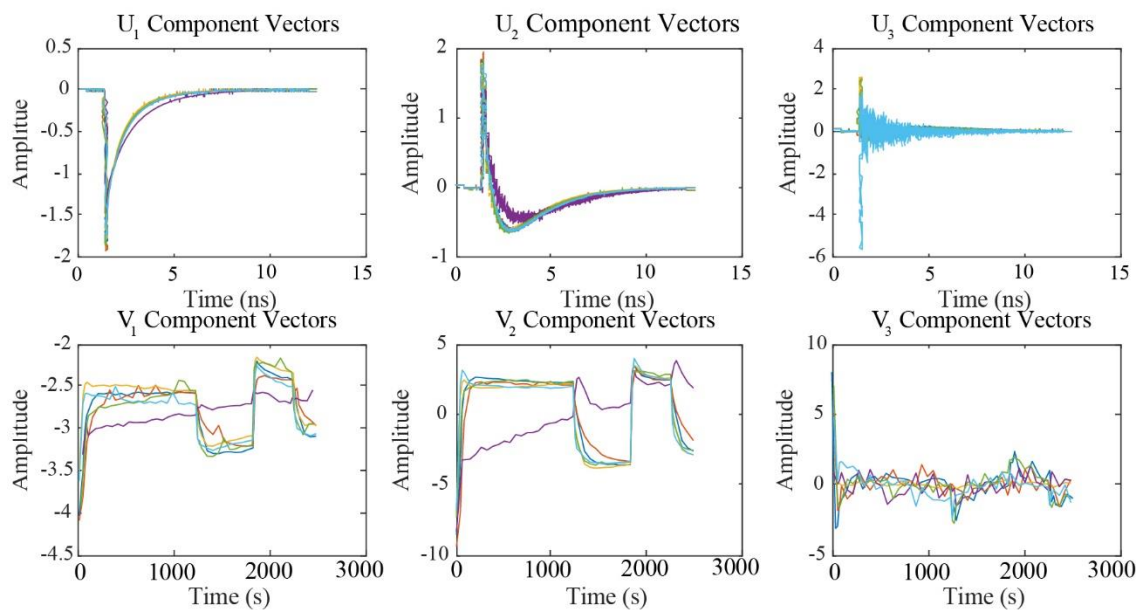


Figure 3.3: Singular value decomposition (SVD) results for the whole dataset. The average U and V vectors corresponding to the largest three singular values are shown for wt (blue), lut2 (red), szl1 (yellow), szl1npq1+NoZEP1 (purple), szl1+NoZEP1 (green), and szl1npq1 (cyan). Using the standard definition of the SVD, $X = USV^*$, the decompositions were performed on matrices, X , composed of the vectors of raw fluorescence lifetime data corresponding to the actinic time points observed for a sample. The resulting decompositions were averaged over 20 samples for each line. In this construction, U is composed of the ultrafast dynamics basis vectors, V is composed of the actinic light acclimation time basis vectors, and S holds the singular values. The first two basis vectors of the ultrafast dynamics basis set clearly exhibit structure; the third exhibits far less structure.

The actinic light acclimation basis set vectors exhibit two components with structure; the third exhibits noise. The singular values corresponding to the sets {U1, V1}, {U2, V2}, and {U3, V3} drop by an order of magnitude between each set. Collectively, the result indicates that fitting more than two components does not extract additional meaningful information, reflecting the tradeoff between limited data collection time of snapshot measurements and the dynamic range of the fluorescence decay curves.

Fluorescence decays were collected from 20 samples per mutant over the course of the light acclimation scheme and fitted to determine the amplitude-weighted average lifetimes. The traces of average lifetimes for the different mutants over the course of acclimation are shown in [Fig. 3.4](#). Mutants containing only a constant high level of lutein (*szl1* and *szl1npq1*) ([Fig. 3.4 C and F](#)) display a rapid overshoot and relaxation to the steady-state quenching level in response to both light acclimation periods. Mutants containing a xanthophyll cycle (*lut2*, which accumulates zeaxanthin via the VAZ cycle, and *szl1+NoZEP1*, which accumulates lutein via the LxL cycle) ([Fig. 3.4 B and E](#)) do not display this overshoot in the first light acclimation period. The *szl1npq1+NoZEP1* strain ([Fig. 3.4D](#)) containing neither xanthophyll cycle does not show any reversible quenching; *wt* plants ([Fig. 3.4A](#)) acclimate faster than *lut2* mutants containing only the VAZ cycle but do not show the initial overshoot of *szl1*.

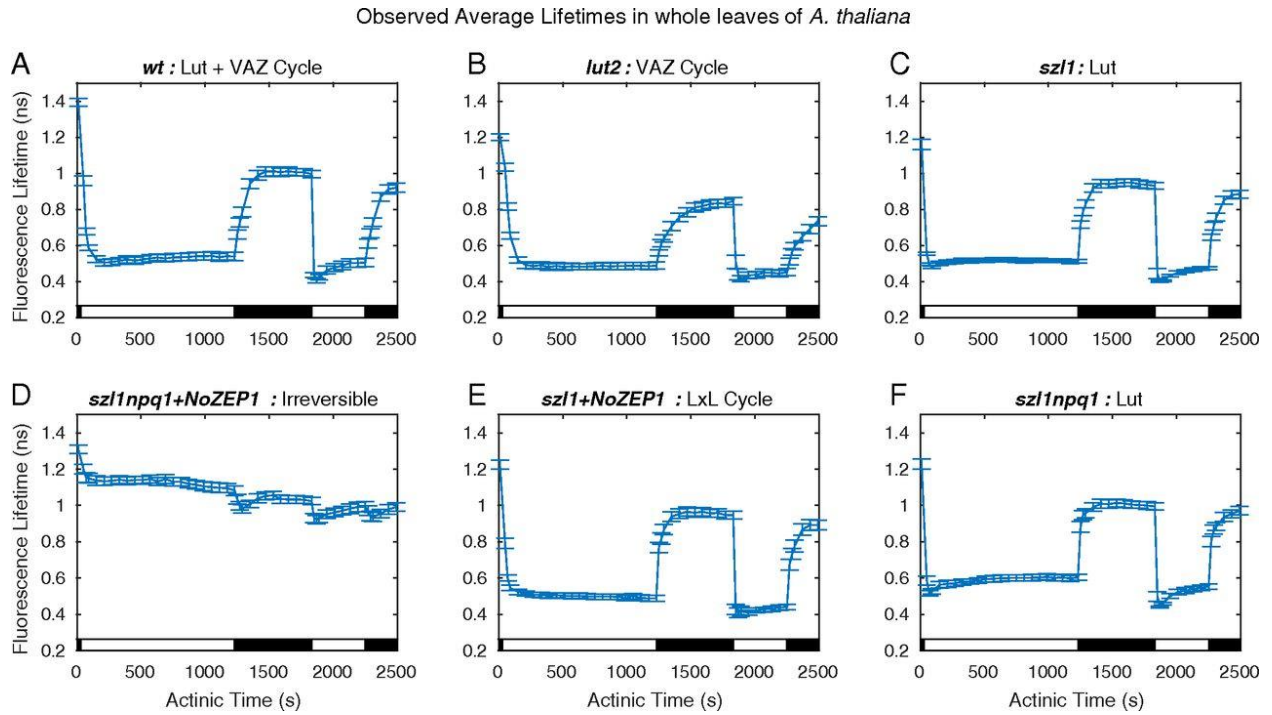


Figure 3.4: Average fluorescence lifetime traces over a two-cycle light acclimation scheme shown by the light and dark bars superimposed on the bottom of each plot for six *A. thaliana* strains. Error bars denote SD for $n = 20$. (A) The *wt* contains lutein and a VAZ cycle to form zeaxanthin in high light conditions. (B) The *lut2* lacks lutein and has an active VAZ cycle. (C) The *szl1* lacks zeaxanthin because of a partially blocked β -carotene biosynthesis pathway and contains more lutein than *wt*. (D) The *szl1npq1+NoZEP1* strain does not have either xanthophyll cycle. (E) The *szl1+NoZEP1* lacks zeaxanthin and contains a nonnative zeaxanthin epoxidase that functions on lutein, converting lutein to lutein epoxide that can be converted back to lutein by native VDE in high light. (F) The *szl1npq1* contains lutein but lacks zeaxanthin because of blocking of the β -carotene biosynthesis pathway and inhibition of VDE.

Modeling

To model the quenching processes observed in the data, a quenching parameter, Q , is calculated from the normalized average lifetimes, τ , allowing for direct comparison of the quenching behavior between

different plant lines. The amplitude-weighted average lifetimes are proportional to fluorescence yield, ϕ , which is given by the ratio of the rate of fluorescence to the sum of the rates of all relaxation processes, including fluorescence, quenching, and other processes, such as energy transfer and intersystem crossing. The expression relating the average lifetimes to the quenching parameter is given in [Eq. 1](#). The rates used for the various processes were obtained from Zaks et al. [38]:

$$\tau \propto \phi = \frac{k_{fluor}}{k_{fluor} + k_{other} + k_{quenching} Q} \quad [1]$$

The parameter Q is a dimensionless modifier of the effective quenching rate and a function of activated PsbS, lutein, zeaxanthin, and other variables. The product of $k_{quenching}$ and Q produces an effective rate of quenching that results in the observed lifetimes. Although previous work has attributed Q to a fraction of activated quenching sites [38], this type of analysis is also valid for more complicated underlying mechanistic details of energy dissipation, such as the alterations to the rate of quenching at an individual site, the density of quenching sites, or multiple types of quenching sites. For simplicity, a single constant rate of quenching at each site, $k_{quenching}$, is assumed, and Q can be considered an effective fraction within these caveats. The particular choice of the value of $k_{quenching}$ does not severely impact the observed behavior of the quenching dynamics but linearly scales the numerical values of Q obtained from the average lifetime and the numerical values of parameters that describe the dynamical behavior. Q is similar to the usual NPQ parameter [2] calculated from, for example, PAM fluorescence traces but is scaled to reflect estimates of the physical processes involved and emphasizes the competition in the experiment between quenching and fluorescence or productive photochemical pathways.

Because the denominator of [Eq. 1](#) is the sum of rates of various processes, using the parameter Q allows for the direct addition and subtraction of various quenching processes within and across mutant strains. In this work, Q is partitioned into a reversible component and an irreversible component based on the observed behavior per [Eq. 2](#):

$$Q = Q_{rev} + Q_{irr} \quad [2]$$

The *szl1npq1+NoZEP1* strain contains no lutein or zeaxanthin and displays nearly monotonically increasing quenching over the course of the experiment. The Q values calculated from *szl1npq1+NoZEP1* lifetimes via [Eq. 1](#) are identified solely as irreversible quenching, Q_{irr} . These values can be subtracted from the values of Q calculated from other strains to isolate the values of reversible quenching, Q_{rev} .

Reversible quenching was modeled using differential equations describing a pair of two-state systems. The systems individually represent lutein- and zeaxanthin-dependent quenching, and each system contains “active” and “inactive” quenching states. The solution to the differential equation for the active quenching states, Q_{active} , gives predicted values of Q_{rev} caused by either lutein or zeaxanthin for comparison with experimental results. The differential equations are simple kinetic rate equations with activation rates given by functions of the variables ΔpH across the membrane, the concentration of activated PsbS, and the concentration of the appropriate xanthophyll(s).

The dynamics of ΔpH and the activation of PsbS and VDE in response to ΔpH were obtained from the model described by Zaks et al. [38]. Their work concluded that the temporal behavior of ΔpH across the thylakoid membrane was insensitive to the detailed parameters of the model of quenching. Therefore, ΔpH is a function solely of time for a given light acclimation scheme and does not depend on the differential equations. Qualitatively, ΔpH has a fast, initial rise on the timescale of seconds on exposure to high light, which then peaks and decays to a steady-state level on the timescale of a few minutes. On initiation of dark relaxation, ΔpH decays from the high-light steady-state level to a dark-adapted steady-state level on the timescale of seconds. The plot of ΔpH in terms of the $[H^+]$ concentration gradient and normalized activity of PsbS and VDE is shown in [Fig. 3.5](#).

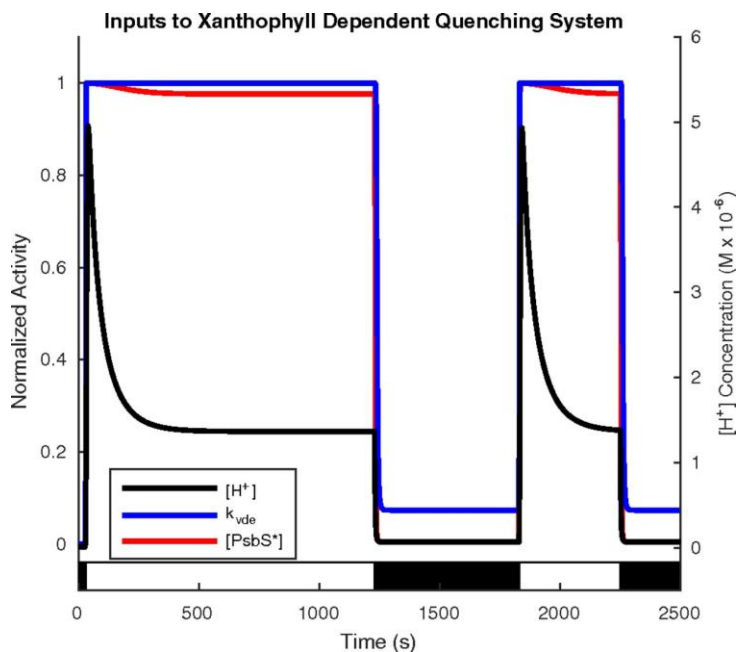


Figure 3.5: Dynamics of thylakoid membrane ΔpH in terms of $[H^+]$ concentration gradient and activation of PsbS and VDE. The black line shows the $[H^+]$ concentration for the light acclimation scheme shown in the light and dark bars superimposed on the plot. It displays a rapid spike on high light exposure followed by a decay to a steady-state value; on dark acclimation, $[H^+]$ concentration rapidly recovers to a dark-adapted quasisteady-state value. The blue line shows the normalized activity of VDE, which is activated on the formation of the ΔpH . The red line shows the normalized activation of PsbS on the formation of the ΔpH . All values were obtained from the model provided in the work by Zaks et al. [38].

The kinetics of the xanthophyll cycles depend on the activation of VDE by ΔpH ; concentrations of the xanthophylls were obtained by fitting a first-order kinetic model with ΔpH -dependent rates of deepoxidation described by Zaks et al. [38] for each relevant mutant to HPLC data to interpolate between measurements. A plot of the wt xanthophyll cycle showing the fractional concentrations of each pigment normalized to the total concentration of the three pigments available for interconversion is shown in [Fig. 3.6](#). Similar fits were performed for the *szl1*+NoZEP1 strain, containing the LxL cycle, showing the expected conversion from lutein epoxide to lutein in response to high light conditions, and the *lut2* mutant, containing the same VAZ cycle as wt ([Figs. 3.7](#) and [3.8](#)).

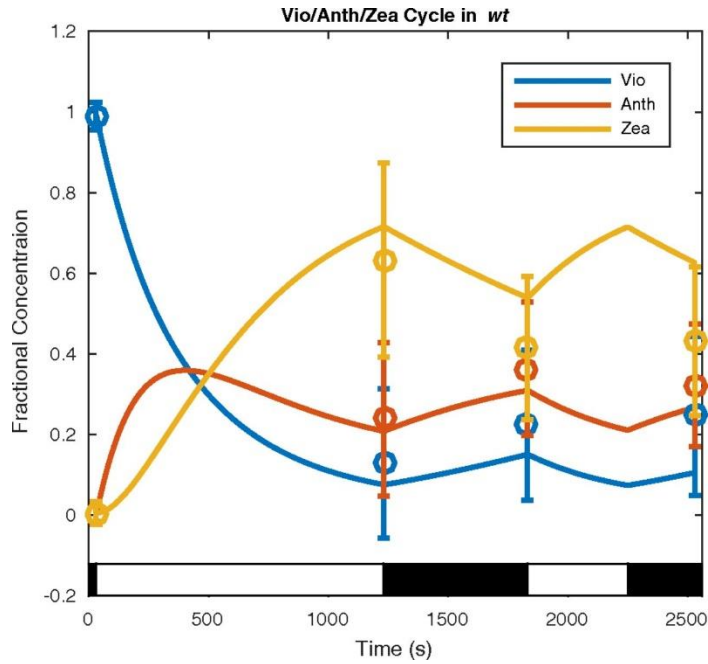


Figure 1.6: Dynamics of the wt xanthophyll cycle in response to the light acclimation scheme shown in the light and dark bars superimposed on the plot fit to HPLC data are shown here. The data are represented by a fit of the fractional concentration of the available pool of violaxanthin (Vio), antheraxanthin (Anth), and zeaxanthin (Zea) to the available pool of xanthophylls determined from analysis of HPLC measurements (Materials and Methods) at four time points (circles with error bars denoting SD for $n = 8$) using a first-order kinetic model with a ΔpH -dependent rate of deepoxidation (solid lines).

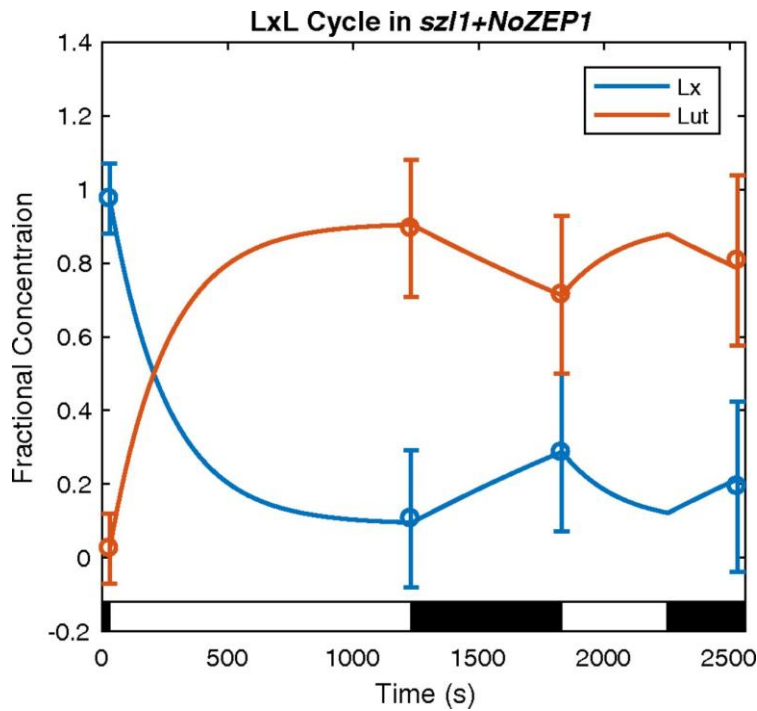


Figure 3.7: Dynamics of the *szl1+NoZEP1* LxL cycle in response to the light acclimation scheme shown in the light and dark bars superimposed on the plot fit to HPLC data are shown here. The data are represented by a fit of the fractional concentration of the available pool of lutein and lutein epoxide to the available pool of xanthophylls determined from analysis of HPLC measurements (Materials and Methods) at four time points (circles with error bars denoting SD for $n = 8$) using a first-order kinetic model with a ΔpH -dependent rate of deepoxidation (solid lines).

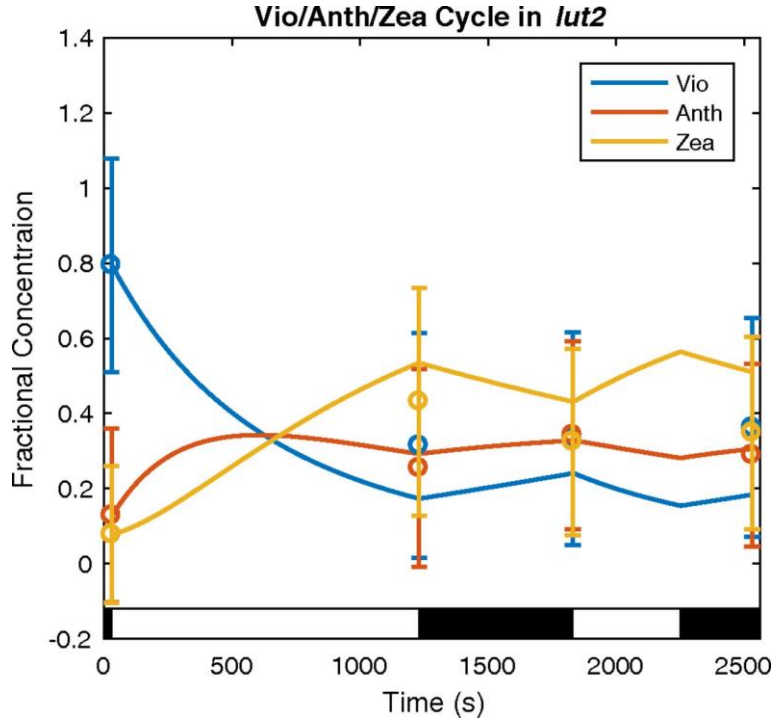


Figure 3.8: Dynamics of the *lut2* VAZ cycle in response to the light acclimation scheme shown in the light and dark bars superimposed on the plot fit to HPLC data are shown here. The data are represented by a fit of the fractional concentration of the available pool of violaxanthin (Vio), antheraxanthin (Anth), and zeaxanthin (Zea) to the available pool of xanthophylls determined from analysis of HPLC measurements (Materials and Methods) at four time points (circles with error bars denoting SD for $n = 8$) using a first-order kinetic model with a ΔpH -dependent rate of deepoxidation (solid lines).

The pool of violaxanthin available for deepoxidation was quantified using Monte Carlo methods to determine the minimum quantity of violaxanthin present using bootstrap resampling of the HPLC data. This technique, described in detail in the section titled, *Materials and Methods*, indicated that $\sim 60\%$ of the measured violaxanthin was unavailable for deepoxidation on the timescales of light acclimation in the experiment, in good agreement with previous work [39]. The same technique was used to remove background antheraxanthin and zeaxanthin present in the dark-acclimated state. The fractional concentration was calculated as the fraction of each individual xanthophyll over the sum of the available pool of xanthophylls.

Lutein-Dependent Quenching

The constant value of lutein in the mutant *szl1*, which contains no zeaxanthin, is the simplest system to model. The constant lutein-dependent reversible quenching model is

$$\frac{d}{dt} Q_{[\text{Lut}]}^{\text{active}} = k_{Q_{[\text{Lut}]}}^{\text{activation}}(t) Q_{[\text{Lut}]}^{\text{inactive}} - k_{Q_{[\text{Lut}]}}^{\text{recovery}} Q_{[\text{Lut}]}^{\text{active}}, \quad [3]$$

$$\frac{d}{dt} Q_{[\text{Lut}]}^{\text{inactive}} = -k_{Q_{[\text{Lut}]}}^{\text{activation}}(t) Q_{[\text{Lut}]}^{\text{inactive}} + k_{Q_{[\text{Lut}]}}^{\text{recovery}} Q_{[\text{Lut}]}^{\text{active}}. \quad [4]$$

The time dependence of $k_{Q[Lut]}^{activation}$ is caused by the time dependence of the activated fraction of PsbS, [PsbS*], which in turn, depends on the ΔpH . The time-dependent activation constant is defined in the form of a Hill equation:

$$k_{Q[Lut]}^{activation}(t) \equiv \frac{[PsbS^*]^n}{K_{[PsbS^*]} + [PsbS^*]^n} k_{Q[Lut]}^{activation} \quad [5]$$

The constants $K_{[PsbS^*]}$ and n describe an equilibrium point and an interaction coefficient of quenching sites activated by PsbS, respectively. Together with $k_{Q[Lut]}^{activation}$, a scaling constant, these constants, which determine the activation, $k_{Q[Lut]}^{activation}$, and $k_{Q[Lut]}^{recovery}$, rates of the quenching, were obtained by fitting solutions of $Q_{[Lut]}^{active}$ in Eq. 3 to values of Q_{rev} calculated from *szl1* lifetime values via Eqs. 1 and 2. A plot of the resulting model, comparing the fit values of $Q_{[Lut]}^{active}$ with the values of Q_{rev} calculated from *szl1* lifetime values and the predicted lifetimes for *szl1*, is shown in 3.9.

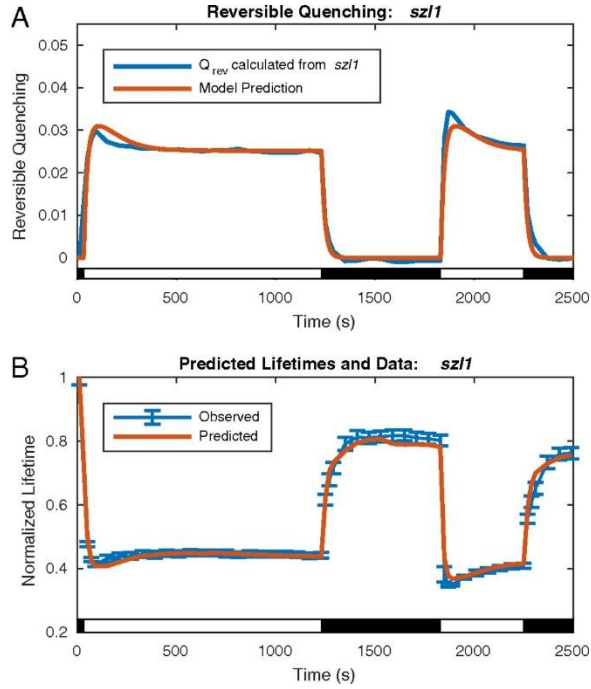


Figure 3.9: Comparison of model to reversible quenching calculated from *szl1* lifetime data via Eqs. 1 and 2 and predicted lifetime with data for *szl1*. (A) The modeled reversible lutein-dependent quenching (red line) compared with the reversible quenching values calculated from *szl1* lifetime data after background subtraction of the irreversible quenching present in *szl1npq1+NoZEP1* (blue line). (B) Normalized fluorescence lifetimes predicted by the model on reconstruction of fluorescence yield from the model for lutein-dependent reversible quenching in *szl1* and the irreversible quenching extracted from *szl1npq1+NoZEP1* (red line) compared with data (blue line; data points with error bars denoting SD for $n = 20$).

The two plots of Q_{rev} and the lifetime values are essentially reciprocals as a consequence of Eqs. 1 and 2. What appears as a slight dip in the lifetime at early acclimation times is reflected in a larger apparent spike, or overshoot of the steady state, in the value of the Q_{rev} . The model is able to describe the initial overshoot of quenching in response to high light, the steady-state level during light exposure, and the recovery in dark. Small discrepancies remain between the light acclimation periods, with the initial overshoot overestimated in the first period and underestimated in the second period. On recovering the predicted lifetimes from the model for *szl1* and the irreversible quenching from *szl1npq1+NoZEP1*, it is apparent that these discrepancies are commensurate in scale with the uncertainty in the data. In this simple system, reversible quenching, Q_{rev} , appears to track directly with the previously predicted ΔpH .

Next, we modeled the *szl1+NoZEP1* strain, which contains the LxL cycle, adding a further complication to account for in our model. In the LxL cycle, lutein epoxide is deepoxidated to lutein in response to the formation of ΔpH . Therefore, in addition to the activation of quenching in response to high light, lutein accumulates. The LxL cycle was verified by HPLC and fit to a first-order kinetic model as discussed above and shown in Fig. 3.7. To account for the impact of the accumulation of lutein on the quenching behavior, the activation rate in the previous model of *szl1* quenching was modified to contain the product of two responses: one to the activated PsbS as shown previously, and a second response to the concentration of lutein, also in the form of a Hill equation. The time-dependent activation rate is redefined as

$$k_{Q_{[Lut]}}^{activation}(t) \equiv \frac{[PsbS^*]^n}{K_{[PsbS^*]} + [PsbS^*]^n} \frac{[Lut]^m}{K_{[Lut]} + [Lut]^m} k_{Q_{[Lut]}}^{activation}, \quad [6]$$

which incorporates an additional $K_{[Lut]}$, an equilibrium value, and m , an interaction coefficient, in the Hill-type response to lutein. A comparison of predicted lifetimes associated with best fit solutions of $Q_{[Lut]}^{active}$ active to values of Q_{rev} , calculated from *szl1+NoZEP1* lifetime data using Eqs. 1 and 2, is shown in Fig. 3.10.

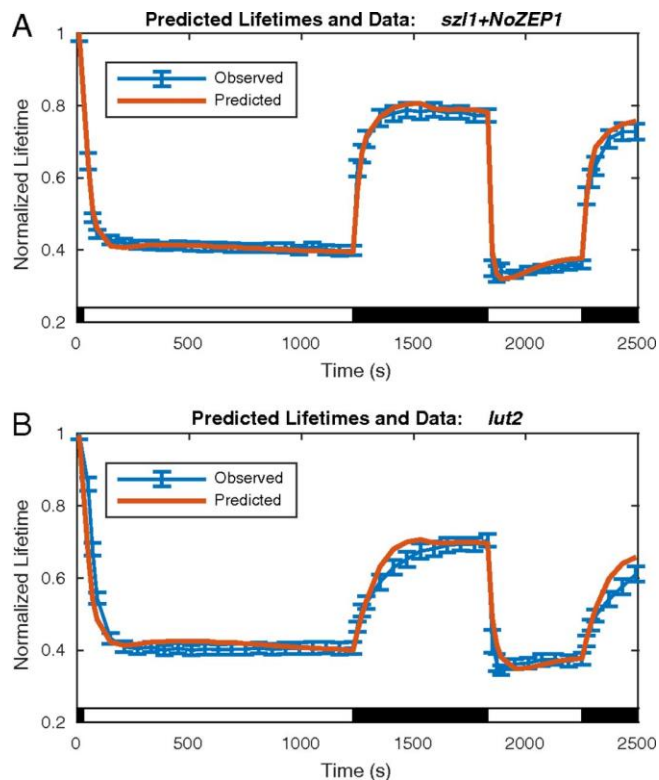


Figure 3.10: Comparison of predicted and observed lifetimes for *szl1+NoZEP1* and *lut2*. (A) Normalized fluorescence lifetimes predicted by the model of reversible quenching for the LxL cycle mutant *szl1+NoZEP1* and irreversible quenching from *szl1npq1+NoZEP1* (red line) compared with *szl1+NoZEP1* lifetime data (blue line; with data points with error bars denoting SD for $n = 20$). (B) Normalized fluorescence lifetimes predicted by the model of reversible quenching for the luteinless VAZ cycle mutant *lut2* and irreversible quenching from *szl1npq1+NoZEP1* (red line) compared with *lut2* lifetime data (blue line; with data points with error bars denoting SD for $n = 20$).

The model captures much of the behavior of the lutein-dependent quenching in *szl1+NoZEP1*: a smooth transition from the dark-acclimated lifetime to a steady-state quenched lifetime in the first acclimation period followed by a sharp spike in the second acclimation period. Because of the additional complicating factor of the accumulation of lutein, the direct correspondence between ΔpH and Q_{rev} seen in *szl1* mutants is obscured. The initial accumulation of lutein suppresses the overshoot that is seen in the second acclimation period, when lutein is present at the outset because of the slower rate of reepoxidation.

Zeaxanthin-Dependent Quenching

The lifetime data show similar characteristics for the LxL cycle strain *szl1+NoZEP1* (Fig. 3.10A) and the luteinless mutant *lut2* (Fig. 3.10B), which only contains the native *A. thaliana* VAZ cycle, suggesting that a similar model can be used to describe zeaxanthin-dependent quenching. The same two-state system, with analogous terms containing an activation rate formed from the product of response to activated PsbS and the xanthophyll and a constant recovery rate, was used but with zeaxanthin substituted for lutein. This construction is sufficient to account for the zeaxanthin- and ΔpH -dependent portion of the quenching response (usually thought of as a portion of qE).

However, on close examination, there is a discrepancy between the reversible quenching behavior of the LxL cycle *szl1+NoZEP1* mutant and the VAZ cycle *lut2* mutant seen in the recovery displayed in the dark. Although the reversible quenching of the LxL cycle *szl1+NoZEP1* strain recovers fully on dark relaxation, the reversible quenching of the VAZ cycle *lut2* mutant does not fully recover on dark relaxation because of the contribution of zeaxanthin-dependent but non- Δ pH-dependent quenching [qZ [24]]. To account for this difference, an additional term is required in the model differential equations. The additional term is independent of $Q_{[Zea]}^{active}$ and linearly dependent on the concentration of zeaxanthin. It carries the opposite sign as the recovery term and shifts the steady-state recovery level when zeaxanthin is present. Although the non- Δ pH-dependent quenching operates on a timescale slower than the Δ pH-dependent quenching, it is still included in the values of Q_{rev} determined from the partitioning scheme. The model system, therefore, is given by

$$\frac{d}{dt} Q_{[Zea]}^{active} = k_{Q_{[Zea]}}^{activation}(t) Q_{[Zea]}^{inactive} - k_{Q_{[Zea]}}^{recovery} Q_{[Zea]}^{active} + k_{qZ} [Zea] \quad , \quad [7]$$

$$\frac{d}{dt} Q_{[Zea]}^{inactive} = -k_{Q_{[Zea]}}^{activation}(t) Q_{[Zea]}^{inactive} + k_{Q_{[Zea]}}^{recovery} Q_{[Zea]}^{active} - k_{qZ} [Zea] \quad , \quad [8]$$

$$k_{Q_{[Zea]}}^{activation}(t) = \frac{[PsbS^*]^n}{K_{[PsbS^*]} + [PsbS^*]^n} \frac{[Zea]^m}{K_{[Zea]} + [Zea]^m} k_{Q_{[Zea]}}^{activation} \quad , \quad [9]$$

where the first two terms, including $k_{Q_{[Zea]}}^{activation}(t)$, are analogous to the model for the LxL cycle *szl1+NoZEP1* strain and the final term accounts for the zeaxanthin-dependent, non- Δ pH-dependent quenching behavior unique to zeaxanthin. The predicted lifetimes associated with fit of values of $Q_{[Zea]}^{active}$ to values of Q_{rev} calculated from *lut2* lifetime values by the same method as previously described are shown in [Fig. 3.10B](#). The fit values again capture the differences between the first and second light acclimation periods, this time caused by the accumulation of zeaxanthin but analogous to the accumulation of lutein. The additional term unique to this model also captures the shift in the recovery level caused by the zeaxanthin-dependent, non- Δ pH-dependent qZ behavior.

Constructing wt Quenching from Components

The zeaxanthin- and lutein-dependent quenching in the mutants containing just one of two xanthophylls allows for a comparison of the ability to quench on a per-lutein or -zeaxanthin basis. Quantities of lutein and zeaxanthin determined from HPLC were normalized to the quantity of Chla, and in turn, the quasisteady-state values of quenching associated with lutein and zeaxanthin were compared on normalizing by the quantity of lutein and zeaxanthin present at quasisteady state. The quasisteady-state quenching values for the lutein-dependent quenching in *szl1*, normalized to the quantity of lutein, were approximately 10 times lower than the quasisteady-state values of zeaxanthin-dependent quenching in *lut2*, normalized to the quantity of zeaxanthin. This analysis indicates that, on average, each zeaxanthin molecule contributes 10 times more to the

overall quenching than each lutein molecule. The difference could be because of either a difference in actual rate of quenching or a difference in the fraction of time in which each molecule is in a quenching state when activated, resulting in a reduced density of quenching sites not accounted for by the concentrations. However, this analysis relies on homogeneous contributions and does not account for potentially nonuniform contributions of the lutein and zeaxanthin molecules (e.g., if only a specific and unique fraction of the lutein molecules present contributes to quenching dynamics, the analysis fails).

One way to test whether the models developed for *szl1* and *lut2* have captured the essence of the quenching process involving these two xanthophylls is to use these models to predict the *wt* response. The zeaxanthin-dependent reversible quenching calculated from *lut2* lifetime measurements using Eqs. 1 and 2, denoted Q_{rev}^{lut2} , and the lutein-dependent reversible quenching calculated from *szl1* lifetime measurements, again using Eqs. 1 and 2, denoted Q_{rev}^{szl1} , were each weighted by the ratio of the average concentrations of the relevant carotenoids present in *wt* relative to the mutant overexpression levels and added to obtain a predicted *wt* quenching value containing the behavior of both lutein- and zeaxanthin-dependent quenching. This value was multiplied by a single common scaling factor, α , to fit the value of Q_{rev} calculated using Eqs. 1 and 2 from *wt* lifetime measurements, denoted Q_{rev}^{wt} . The expression for the predicted *wt* reversible quenching is

$$Q_{rev}^{wt} = \alpha \left(\frac{\langle [Lut] \rangle_{wt}}{\langle [Lut] \rangle_{szl1}} Q_{rev}^{szl1} + \frac{\langle [Zea] \rangle_{wt}}{\langle [Zea] \rangle_{lut2}} Q_{rev}^{lut2} \right) \quad [10]$$

The lutein and zeaxanthin ratios in Eq. 10 were determined from the HPLC data to be 0.67 and 0.24, respectively. The scaling factor, α , was fitted to 1.37.

Plots of the reconstructed *wt* quenching parameter, Q_{rev}^{wt} , are compared with values calculated from *wt* lifetime data via Eqs. 1 and 2 in Fig. 3.11A; the *wt* lifetimes predicted from the reconstruction are compared with the measured *wt* lifetime data in Fig. 3.11B. The reversible quenching values, Q_{rev}^{szl1} and Q_{rev}^{lut2} , calculated from lifetime data from *lut2* and *szl1* are plotted after scaling by the ratio of the concentrations and α together with their sum, the predicted reversible quenching for *wt*, Q_{rev}^{wt} . The predicted value of the reversible quenching for *wt* from the components agrees well with the value calculated directly from the *wt* lifetime data. The lifetimes resulting from these values in Fig. 3.11B show that the variation from the observed *wt* lifetime data is on the order of the uncertainty of the measurement.

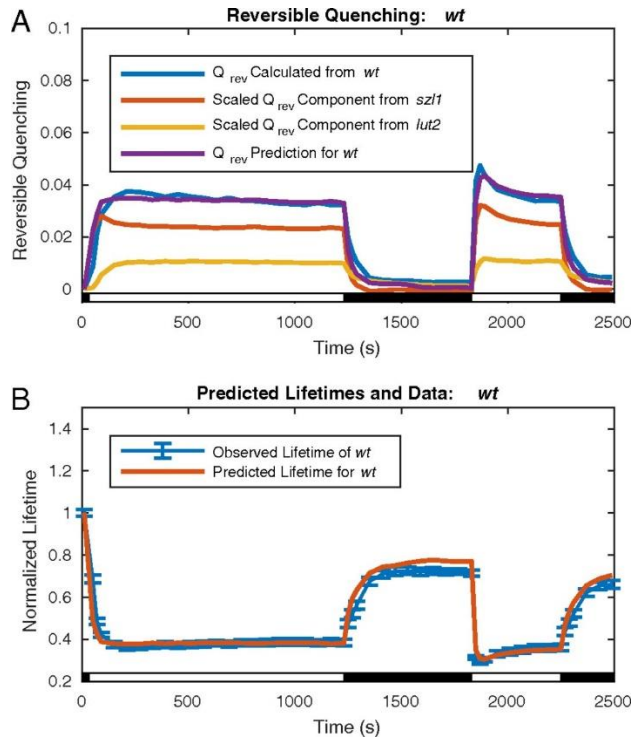


Figure 3.11: Comparison of wt reversible quenching calculated from lifetime data via Eqs. 1 and 2 with predicted values calculated from *szl1* and *lut2* mutant lifetime data via Eq. 10 and corresponding lifetimes. (A) Reversible quenching calculated via Eqs. 1 and 2 from wt lifetime data (blue) agrees well with the predicted reversible quenching (purple) obtained from *szl1* (red) and *lut2* (yellow) contributions via Eq. 10 (in the text). (B) The wt lifetimes (red) predicted from the wt reversible quenching obtained via Eq. 10 and irreversible quenching from *szl1npq1*+NoZEP1 are within the error of the observed lifetimes of wt (blue; including error bars indicating SD for $n = 20$).

The success of our approach in reproducing both the steady-state quenching and the quantitative value of the overshoot in the second light acclimation period with only a single common scaling factor has several implications. First, the finding supports the previous analysis of the relative average contributions to quenching of each xanthophyll. Linear scaling by the xanthophyll concentrations reproduces the observed wt quenching across a range of quenching values, suggesting that, for the range of concentrations found in the mutants studied, the contributions of each molecule in the xanthophyll pool are indeed homogenous. Microscopically, achieving homogeneous contributions corresponds to a regime where the activation of quenching sites is limited by the availability of the xanthophylls and does not correspond to a regime limited by binding sites capable of quenching. In the alternative regime, the ratio of participating sites to concentration of the xanthophylls would not scale linearly across the range of observed quenching values. Instead, the quenching would scale linearly with the available binding sites. At yet higher concentrations of xanthophylls (e.g., in mutants that more strongly overexpress the xanthophylls), these regimes may no longer hold true.

Second, despite correctly predicting the ratio between the steady-state and overshoot quenching on scaling the contributions by the average concentrations of the xanthophylls, the additional common scaling factor, α , is still required to quantitatively predict the observed wt quenching values. There are several possible explanations for the scaling factor. For example, the presence of both xanthophylls may increase the density of quenching sites for a given concentration of each xanthophyll because of more efficient binding of the correct xanthophyll in certain sites.

Furthermore, there is evidence that substitution at lutein or zeaxanthin sites occurs in mutants lacking the preferred xanthophyll [36]. On substitution, changes in quenching rate caused by the substitute xanthophyll could result in reduced quenching for a given density of quenchers in mutants lacking the correct xanthophyll. Finally, in sites where zeaxanthin and lutein are in close proximity to both one another and a chlorophyll, the combined presence may work to cooperatively increase the quenching rate beyond the rates of quenching possible in the presence of either xanthophyll individually [29], [30]. However, because both lutein-dependent quenching and zeaxanthin-dependent quenching are able to operate independently, it seems that zeaxanthin is unlikely to function solely as an allosteric regulator. Reasonable physical models [40] could help evaluate the extent that various possibilities could explain the behavior observed for *wt* quenching. Determining the density of quenching sites required to predict the lutein- and zeaxanthin-dependent quenching and comparing with *wt* would indicate which, if any, of these effects is consistent with the observed behavior, but it may be difficult to decouple the product of quenching rate and quenching site density, thus requiring additional constraints to separate these quantities.

Conclusion

There are multiple mechanisms that contribute to NPQ in *wt* plants. We have shown the use of NPQ data from various mutants to isolate specific contributions from lutein and zeaxanthin and use these to reconstruct the response of the *wt*, which results from several (two or more) different contributions. The success of the approach of Kromdijk et al. [4] in increasing plant productivity by changing expression levels of VDE, zeaxanthin epoxidase, and PsbS indicates that optimizing the rates of violaxanthin to zeaxanthin and lutein epoxide to lutein interconversion along with the concentration of PsbS is a viable route to increased photosynthetic yield. The kinetic model developed here will enable optimal values of concentrations and yields to be explored and tested in real field trials to determine if the 15% increase described in *Nicotiana* (tobacco) [4] can be further improved on. From the perspective of refining the model, the distinct differences observed between the two light acclimation periods suggest that varying frequency periodic illumination periods could enable the separation of the multiple quenching processes that occur on different timescales, but for example, all depend on the presence of zeaxanthin.

Materials and Methods

Plant Material and Growth Conditions

A. thaliana WT plants (Col-0) and mutant plants *lut2*, *szl1*, *szl1npq1*, *szl1+NoZEP1*, and *szl1npq1+NoZEP1* were germinated on plates, transplanted to pots, and grown in growth chambers under 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ on a 10-h day, 14-h night schedule at 23 °C. Mutant descriptions are shown in [Table 3.1](#). The LxL cycle strains [35] were screened for homozygosity on plates containing Basta. The NPQ phenotype of these mutants, which contain zeaxanthin epoxidase from *N. oceanica* [41], was confirmed using pulse-amplitude modulated (PAM) fluorescence using an IMAGING-PAM M-series (Heinz Walz) instrument to monitor NPQ capacity. Lastly, HPLC data confirmed the presence of lutein epoxide, which is conclusive evidence of the functionality of the nonnative zeaxanthin epoxidase from *N. oceanica* in *A. thaliana* plants. All plants were between 6 and 9 weeks of age at the time of experiments, and all measurements were completed before the stage of bolting as described previously [42].

TCPSC Measurements

Each sample set was made up of 20 whole leaves from each respective genotype. Before TCSPC snapshot experiments, plants were dark-acclimated for 30 min to ensure that the relevant xanthophylls in plants containing xanthophyll cycles were in their inactive, epoxidized state at the start of the experiment. All plant lines except for *lut2* were exposed to $745 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ during high light periods. *Lut2* plants were subjected to $620 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ during periods of high light to achieve similar quenched average lifetimes as *szl1+NoZEP1* exposed to $745 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ high light periods to facilitate comparison of the shape of the two decay curves at intensities where the average lifetimes of the two mutants are the same. The decay curves of *lut2* and *szl1+NoZEP1* were very similar, as were the fitted parameters, limiting the extraction of any mechanistic implications. However, the change from 745 to $620 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ should not significantly impact the analysis reported in this work. In contrast to PAM traces, data from TCPSC measurements are relatively insensitive to changes in high light intensity, because time resolving the fluorescence eliminates sensitivity to nonquenching processes, including chloroplast avoidance [42]. The remaining differences that could have implications for the reconstruction of *wt* from components were subsequently accounted for by normalization of the average lifetimes and the resulting zeaxanthin concentration in *lut2* compared with *wt*. Plants were kept under $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light when not being dark-acclimated, and no plant was dark-acclimated more than once during any 1.5-h period.

After initial dark acclimation, TCSPC snapshots were collected during two cycles of high light followed by dark relaxation. The first cycle consisted of 20 min of high light followed by 10 min of darkness. The second cycle, immediately after the first cycle, consisted of 7 min of high light followed by 3 min of darkness. Leaves were removed from dark-acclimated plants immediately before TCSPC experiments and placed in a home-built holder that allows the leaf surface to be mostly exposed to air to avoid overheating and drying during the experiment and has a small well to hold water for the petiole to take up during the experiment as described previously [42].

The TCSPC setup was similar to the ones described in the works by Sylak-Glassman et al. [42] and Amarnath et al. [43]. A 532-nm Coherent Verdi G10 diode laser pumped an ultrafast Ti:Sapph Coherent Mira 900f oscillator with the birefringence adjusted, such that the center wavelength was 840 nm with an FWHM of ~ 9 nm. The 840-nm output pulses from the Mira were then frequency doubled to 420 nm using a beta barium borate crystal to excite the Soret band of Chla. Before the sample area, the beam was split by a beam splitter, so that a small portion was sent to a sync photodiode and acted as a reference pulse for the TCSPC measurements, whereas the remainder was sent to the sample area, where it was incident on the leaf. Data were acquired using a Becker & Hickl SPC-850 data acquisition card in conjunction with the appropriate Becker & Hickl software and the sequence of shutter operations executed using LabView. The portion of the beam that reached the sample was incident on the leaf at a 70° angle to the adaxial side of the leaf. The average power of the laser at the sample was 1.75 mW, corresponding to about $1,800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light, which is enough to reach saturation of closed reaction centers [44], with a pulse energy of 19.8 pJ. A monochromator (HORIBA Jobin-Yvon; H-20) set to transmit 684 ± 8 nm was placed before the MCP PMT detector (Hamamatsu R3809U MCP-PMT) to selectively observe fluorescence from the Q_y band of Chla molecules in PSII. The actinic light source was a Leica KL1500 LCD with dual gooseneck fiber optic cables to allow for acclimation of two samples to the same light conditions simultaneously.

The detector was cooled to $-30\text{ }^{\circ}\text{C}$, and the gain was set to 94% (controlled by Becker & Hickl software), yielding an instrument response function with an FWHM of 36–38 ps. Each fluorescence lifetime snapshot consisted of a 1-s period of laser exposure and data collection. The lifetime data were partitioned into five 0.2-s steps. During subsequent analysis, the step with the longest average lifetime, which corresponds to the step with the highest fluorescence yield in a PAM trace, was retained as the measurement with the reaction centers closed, similar to ref. [42].

Data Analysis

To avoid the need for additional physically meaningless lifetime components, each curve was fitted individually to a biexponential function using nonlinear least squares analysis rather than aligning the curves according to their maxima and summing them to average the data before fitting. This method also allowed for the step with the reaction center closed to be chosen before any averaging was done, ensuring that the reaction centers were closed in each leaf for each snapshot rather than just on average. SDs on each fit parameter were calculated from the Jacobian, and a reduced χ^2 value was calculated for each fit to confirm goodness of fit. Residuals of several curves from each dataset were examined as an additional check on goodness of fit. Furthermore, singular value decomposition revealed only two components, validating the use of a biexponential function to fit the data (Fig. 3.3).

After each curve was fitted to a biexponential decay function, the amplitude-weighted average lifetime associated with each decay was calculated, and the uncertainty associated with it was determined from the SD of the fit parameters. Next, the step with the longest lifetime was chosen as the step with the reaction centers closed to saturation. At this point, the rest of the data were discarded, and only the data collected when the reaction centers were identified as closed to saturation were further analyzed. The fits for each snapshot were used to calculate uncertainty-weighted averages of each of the components across 20 leaf samples. Because of normal variability in fluorescence yield from different leaves, it was necessary to normalize the amplitudes associated with the decay times to sum to one to make them comparable across all samples. The average amplitude-weighted lifetime across 20 samples was calculated for each snapshot from the uncertainty-weighted averages of the fit parameters, and the uncertainties on the parameters were then used to calculate the uncertainty on the average amplitude-weighted lifetime for each decay. Decay time, amplitude, and average amplitude-weighted lifetime for each snapshot/decay were then bootstrapped by examining the variation across resampling of the data from 20 samples collected for each snapshot during TCSPC. Because the amplitudes were normalized to sum to one, their uncertainties cannot be decoupled. The uncertainty on the amplitude of the shorter decay time was calculated first, and the uncertainty on the amplitude of the larger decay time was then back calculated. The SDs were obtained by calculating a 68% confidence interval from the resampled dataset generated during bootstrapping. These confidence intervals were used to generate error bars on the traces of amplitude-weighted average lifetimes during TCSPC measurements.

Monte Carlo Methods to Determine Available Xanthophyll Pools

To estimate the fraction of the detected xanthophylls available to undergo deepoxidation, Monte Carlo methods were used to determine a constant background pool. Many samples of time series for each xanthophyll were generated from a normal distribution using the mean and SD observed from the measurements at each point in the light acclimation scheme. The minimum value of the concentration of

each xanthophyll across a sample time series was selected as the value of background xanthophyll in the time series. For, for example, violaxanthin, the minimum within a single time series usually occurred after 20 min of light acclimation. The mean minimum values were subsequently determined and identified as the portion of the measured xanthophyll concentrations that did not contribute to the kinetic behavior of deepoxidation and reepoxidation observed.

Model Estimation

Models of the kinetic behavior of the carotenoids and quenching were fit to HPLC data points and values of reversible quenching described previously. The nonlinear greybox estimation tools provided in MATLAB were used to determine best fit values of the parameters by evaluating the normalized mean square error expressed as a percentage. Sets of parameters were initialized on a grid to determine global best fits for each model. Because the algorithm requires equally spaced time points, when a missing time point was required as in the case for the carotenoid kinetic modeling, a dummy point was inserted and allowed to vary by resetting to the predicted value of the fit and repeating until the results converged. The normalized mean square error values, expressed as percentages, were greater than 80% for each mutant model fit and 68% for the fit of the *wt* model to data. Values for the carotenoid fit were all greater than 60% for the active xanthophyll, but because of the small number of time series data points, the fits to HPLC data are only valuable as estimates; 0% corresponds to a straight line at the mean value of the data, and 100% is a perfect fit.

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Chapter 4: Fluorescence lifetime studies on *Arabidopsis thaliana* mutants reveal a novel quenching mechanism localized to LHCII and modulated by a system comprised of the SOQ1 and OTK1 proteins, and a chloroplast lipocalin, AtCHL

Collaborations: This work was done in collaboration with Cynthia Amstutz in the lab of Kris Niyogi in the Plant and Microbial Biology Department at UC Berkeley. The work is strongly connected to and often references work done in the same lab currently by Alizée Malnoë, and previously by Matthew Brooks.

Introduction

As described in chapter 1, NPQ is comprised of several subcomponents which have historically been categorized according to their induction and relaxation rates. The rapidly reversible component, qE, turns on and off in seconds, makes up the majority of the NPQ response, is triggered by ΔpH , and requires PsbS, zeaxanthin and violaxanthin deepoxidase (VDE)[1]–[5]. A more slowly induced component called qZ, reverses in minutes, is dependent on zeaxanthin, does not require ΔpH once zeaxanthin has accumulated [6], and is likely catalyzed by PsbS [7]. The slowest process is called qI and takes hours to relax [2]. While it's the least understood of the NPQ components, qI is thought to be associated with, but cannot fully be accounted for, by PSII reaction center damage [8], [9]. Lastly, qT is a process by which antenna complexes are shuffled around in order to balance the excitation of PSI and PSII under light conditions favoring excitation of one or the other photosystem [2]. Although qT is an important process when plants experience changes in light quality (color), it does not contribute significantly to the response of vascular plants under high light conditions [10] and will not be discussed further in this work. The components and their defining characteristics are described in table 4.1 below.

NPQ Component	Defining Characteristics
qE	Rapidly reversible (seconds) - makes up majority of NPQ - ΔpH triggered - PsbS required - Zeaxanthin required - VDE required.
qZ	-Reversed in minutes - Zeaxanthin required - ΔpH not required once Zeaxanthin accumulates - PsbS catalyzed
qI	-Slowest relaxation time (hours) - least understood - associated with PSII damage but damage cannot account for total qI
qT	-Response to PSI or PSII preferential excitation - requires STN7 kinase - LHCII antenna migrate to balance excitation across PSI and PSII

Table 4.1: Table of NPQ components and their characteristics

Since qE requires PsbS, the PsbS-less mutant, *npq4* [11] provided a tool to investigate NPQ components other than qE, the best understood and thus far, the most studied component of

NPQ. Through chemical mutagenesis of the *npq4* mutant, Brooks et al. discovered a new mutant, *soq1 npq4* which showed partial recovery of NPQ capacity in the absence of PsbS [12]. Removal of the *npq4* background allowed for the study of the quenching process employed by these mutants in the presence of PsbS and the quenching components dependent upon it. The single mutant *soq1* displayed additional quenching above that observed in *wt* plants, indicating that the novel form of quenching still occurs in the presence of other, PsbS-dependent mechanisms. The *soq1* mutant is named for the protein it lacks, called SOQ1, or Suppressor of Quenching 1. The SOQ1 protein is named after its function of suppressing quenching, meaning in the absence of the SOQ1 protein, additional quenching is observed. The newly discovered form of quenching was then further categorized via chemical treatments and crosses with other mutants. Treatment with nigericin, which eliminates ΔpH , did not abolish the additional quenching observed in *soq1* plants relative to *wt* plants, confirming the earlier conclusion that the new form of quenching is unrelated to qE. Knocking out VDE eliminates zeaxanthin accumulation but has no impact on the additional quenching observed in *soq1* indicating the quenching is unrelated to qZ. Crossing *soq1* with the STN7 kinase knockout (which cannot phosphorylate LHCII and therefore cannot engage in state transitions, or qT) also had no impact, proving that *soq1* quenching isn't related to qT. Treatment with lincomycin, which inhibits the PSII repair cycle, revealed an intact, robust repair cycle in *soq1* eliminating the possibility that the quenching is due to an accumulation of damaged PSII units that act as quenchers, the most common explanation for qI. However, as stated above, there are not enough damaged PSII complexes present in *wt* plants to account for the entirety of qI [9]. The *soq1* mutant exhibits a fast decline in average fluorescence lifetime in the first 2.5 minutes, followed by a slow linear decline in average fluorescence lifetime during the remaining illumination period. However, the timescale of the longer decay component in *npq4* depends on SOQ1 and the shorter decay is unaffected by SOQ1, leading to the conclusion that *soq1*-type quenching is a novel, as-of-yet uncharacterized form of qI quenching. Further characterization by Alizée Malnoë, in the Niyogi lab, points toward the likely location of SOQ1 dependent quenching to the peripheral antenna of PSII and the requirement of the lumen localized chloroplast lipocalin CHL for this quenching to occur [13]. The *chl* allele is a knockout mutant for the lipocalin CHL. *soq1 chl* does not display additional quenching compared to *soq1*.

Several experiments suggest SOQ1 dependent quenching likely occurs in antenna complexes. First, trends in the pulse amplitude modulated (PAM) fluorescence yield data show a decrease in F_0' which is often associated with thermal dissipation in antenna complexes [12], [14], [15]. Second, Malnoë et al. demonstrated that Chl *b*, which is most abundant in the major light harvesting complex of PSII (LHCII), is required for SOQ1 dependent quenching by crossing the *soq1* mutant with a *chlorina* mutant lacking Chl *b*. In the absence of Chl *b*, the additional quenching was abolished suggesting LHCII is required for SOQ1 dependent quenching to occur. Lastly, separation of the photosynthetic complexes from cold and high light treated *soq1* and *soq1 chl* plants by gel filtration revealed that even after somewhat invasive techniques are applied, the LHCII trimers from *soq1* are quenched exhibiting a 32% lower fluorescence yield relative to LHCII trimers from *soq1 chl*, while the other antenna complexes and pigment protein complexes are not. The SOQ1 protein is a large multi-domain protein located inside the thylakoid membrane with a C-terminus extending into the thylakoid lumen containing the Trx-like domain (necessary to suppress quenching) and an NHL β -propeller domain, and a single transmembrane α -helix separating the Trx and NHL domains from a stroma-exposed haloacid

dehalogenase-like hydrolase (HAD) domain [12]. Trx motifs are characterized by two vicinal cysteine residues that catalyze a thiol-disulfide exchange reaction to either oxidize or reduce their substrates [16]. But what is the substrate SOQ1 acts on to suppress quenching? In order to try to answer this question, Alizée Malnoë, in the Niyogi lab, did a suppressor screen of *soq1 npq4*, obtaining two mutations that recovered the *npq4* quenching phenotype (no qE). Whole genome sequencing found mutations in the gene encoding a chloroplastic lipocalin (AtCHL). Lipocalins are proteins with a high affinity for small, hydrophobic molecules. AtCHL is about 35kDa, is located in the thylakoid lumen, accumulates under drought and high light stress, and prevents lipid peroxidation [17]. PAM fluorescence yield traces on several mutants revealed that AtCHL is required for SOQ1 modulated quenching. Malnoë et al. suggest a working model of how SOQ1 and AtCHL work together to modulate quenching: under normal conditions, SOQ1 inhibits AtCHL activity, preventing it from activating quenching sites while in the absence of SOQ1, AtCHL is active and quenching sites are produced.

The chemical mutagenesis of *soq1 npq4* also revealed a new mutant that is constitutively quenched, called *soq1 npq4 otk1*. The new mutant has been characterized by Cynthia Amstutz in the Niyogi lab and displays large grana, small stroma lamellae, over stacking of the grana thylakoid membranes, a slow growth phenotype, and an extremely short fluorescence lifetime regardless of light, or dark adaptation status [18]. Next, the *npq4* background was eliminated and the double mutant *soq1 otk1-1* studied (*otk1-1* is a point mutation that impairs the function of OTK). Similar to the *soq1 npq4 otk1* mutant, *soq1 otk1* displays large grana and small stroma lamellae, is constitutively quenched with a very short lifetime and has a slow growth phenotype. The grana of *soq1 otk1* are overstacked like those of *soq1 npq4 otk1* but to a lesser extent. Analysis of the single mutant *otk1* revealed that they grow, fluoresce and quench like *wt* plants demonstrating that the observed phenotype in *soq1 otk1* is a result of the combined impact of the two mutations and not one or the other alone. This was further confirmed by crossing the single *soq1* and *otk1-3* (knockout allele) mutants to recover the severely quenched *soq1 otk1* phenotype. OTK1 is an atypical/extended short chain dehydrogenase/reductase (SDR) (atypical because it's missing a Tyr residue), and has been annotated as putative sugar epimerase or to have possible hydroxysteroid dehydrogenase activity. Moreover, OTK1 is a GreenCut protein, CGLD13 meaning it is conserved in the green lineage and diatoms. OTK1 is a small, soluble protein, localized in the stroma and possibly associated to the stroma lamellae. Mutants with *soq1 otk1* background lacking AtCHL resemble *wt* and *soq1 chl*, indicating that the novel quenching mechanisms observed in *soq1* and *soq1 otk1* most likely occur by the same process, likely also in the LHCII complexes. Additional evidence suggests *soq1 otk1* quenching occurs in LHCII: *soq1 otk1* has more antenna pigments and fewer reaction center pigments but treatment with DCMU does not cause significant increase in fluorescence yield indicating a small functional antenna size. These two observations together suggest that there are many antenna complexes in the mutant but most of them are in a quenched state or are not energetically connected to the PSII reaction centers. Additionally, some evidence suggests *soq1 otk1* accumulates more MGDG, a lipid associated with increased LHCII aggregation quenching, than *wt* [19]. Lastly, *soq1 otk1* displays low 77K fluorescence from PSII and LHCII trimers near 684 nm. In order to confirm that *soq1 otk1* quenching occurs in LHCII, a TCSPC experiment was designed to excite LHCII trimers preferentially by exciting the Soret band of Chl *b* in addition to the typical measurements upon excitation of Chl *a* at 420 nm.

Materials and Methods

TCPSC Measurements

Each sample set was made up of 10 whole leaves from each respective genotype. Before TCSPC snapshot experiments, plants were dark-acclimated for 30 min to ensure that the relevant xanthophylls were in their inactive, epoxidized state at the start of the experiment. In contrast to PAM traces, time resolved fluorescence eliminates sensitivity to nonquenching processes, including chloroplast avoidance [20]. No plant was dark-acclimated more than once during any 1.5-h period. After initial dark acclimation, TCSPC snapshots were collected in the dark. Leaves were removed from dark-acclimated plants immediately before TCSPC experiments and placed in a home-built holder that allows the leaf surface to be mostly exposed to air to avoid overheating and drying during the experiment and has a small well to hold water for the petiole to take up during the experiment as described previously [20]. The TCSPC setup was similar to the ones described in the works by Sylak-Glassman et al. [20] and Amarnath et al. [21]. A 532-nm Coherent Verdi G10 diode laser pumped an ultrafast Ti:Sapph Coherent Mira 900f oscillator with the birefringence adjusted, such that the center wavelength was either at 840 nm or 896nm with an FWHM of ~ 9 nm. The 840 or 896-nm output pulses from the Mira were then frequency doubled to 420 nm or 447 nm respectively, using a beta barium borate crystal to excite the Soret band of Chl *a* or Chl *b*. Before the sample area, the beam was split by a beam splitter, so that a small portion was sent to a sync photodiode and acted as a reference pulse for the TCSPC measurements, while the remainder was sent to the sample area, where it was incident on the leaf. Data were acquired using a Becker & Hickl SPC-850 data acquisition card in conjunction with the appropriate Becker & Hickl software and the sequence of shutter operations was executed using LabView. The portion of the beam that reached the sample was incident on the leaf at a 70° angle to the adaxial side of the leaf. The average power of the laser at the sample was 1.75 mW, corresponding to about $1,800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light at 420 nm and about $1,900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 447 nm, which is enough to reach saturation of closed reaction centers [22], with a pulse energy of 19.8 pJ. A monochromator (HORIBA Jobin-Yvon; H-20) set to transmit $\lambda \pm 8$ nm was placed before the MCP PMT detector (Hamamatsu R3809U MCP-PMT) and the detection wavelength was cycled from 680-760 nm, taking ten snapshots at each wavelength and completing a full data set for each leaf measured. Such a set of measurements takes about 45 minutes, during which the leaf remains in the sample holder. The detector was cooled to -30°C , and the gain was set to 94% (controlled by Becker & Hickl software), yielding an instrument response function with an FWHM of 36–38 ps. Each fluorescence lifetime snapshot consisted of a 1-s period of laser exposure and data collection. The lifetime data were partitioned into five 0.2-s steps. During subsequent analysis, the step with the longest average lifetime, which corresponds to the step with the highest fluorescence yield in a PAM trace, was retained as the measurement with the reaction centers closed, similar to ref. [20]. In this experiment, the excitation wavelength was tuned first to 420 nm to excite the Soret band of chl *a*, and then to 447 nm in an attempt to excite the Soret band of chl *b*. Although a longer wavelength would be more appropriate, the Ti:Sapph laser used in this setup is unable to mode-lock at longer wavelengths without altering the optics inside the cavity. Many detection wavelengths were explored but a select few are discussed here.

Data Analysis

The data were fit using Picoquant's Fluofit software via reconvolution using the Maximum Likelihood Estimation method in order to ensure minimal ($< 10\%$) standard deviations when fitting data with relatively few counts in the maximum count bin [23]. Residuals of several curves from each dataset were examined as an additional check on goodness of fit. After each curve was fitted to a biexponential decay function, the amplitude-weighted average lifetime associated with each decay was calculated. Next, the step with the longest lifetime was chosen as the step with the reaction centers closed to saturation. At this point, the rest of the data were discarded, and only the data collected when the reaction centers were identified as closed to saturation were further analyzed. The average lifetime values for each particular detection wavelength were averaged across the ten samples. Because of normal variability in fluorescence yield from different leaves, it was necessary to normalize the amplitudes associated with the decay times to sum to one to make them comparable across all samples.

Results

Fig. 4.1 shows the quenched fluorescence lifetime decay of the *soq1 otk1* mutant and the average lifetime measured at 420 nm excitation and 680 nm detection wavelengths.

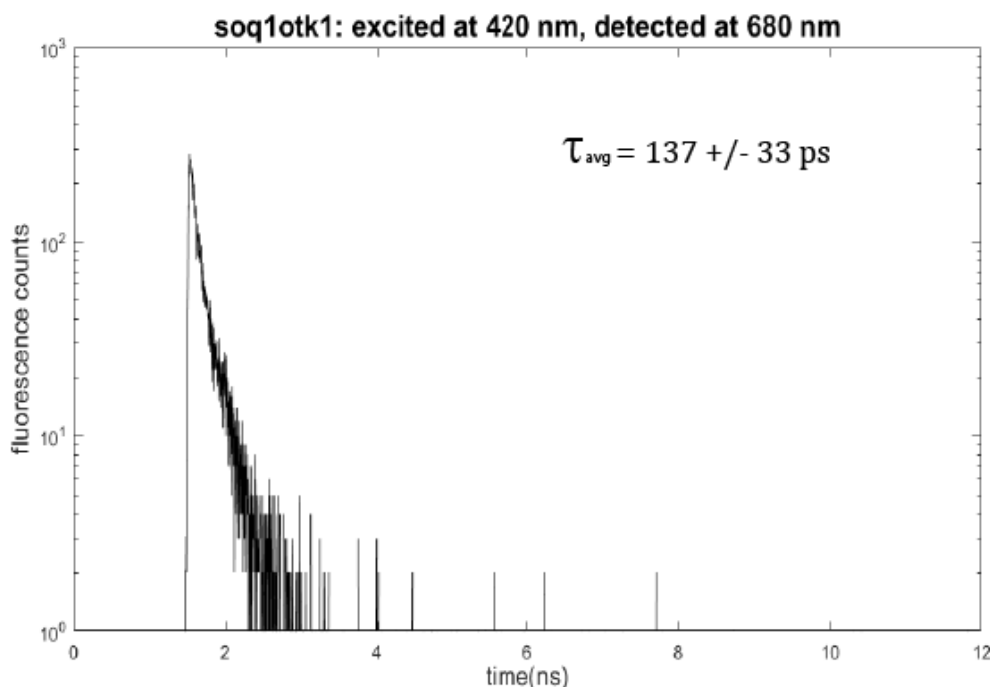


Figure 4.1: Fluorescence lifetime decay of *soq1 otk1* excited at 420 nm, detected at 680 nm. The measured lifetime is 137ps $\pm$ 33ps.

Emission spectra of chl *a* and *b* in solution, showed that excitation at 474 nm and 450 nm achieved similar increases in fluorescence emission in the 440 nm to 460 nm range associated with chl *b* fluorescence (Fig 4.2), suggesting that a significantly higher portion of chl *b* molecules are excited at 450 nm relative to 420 nm.

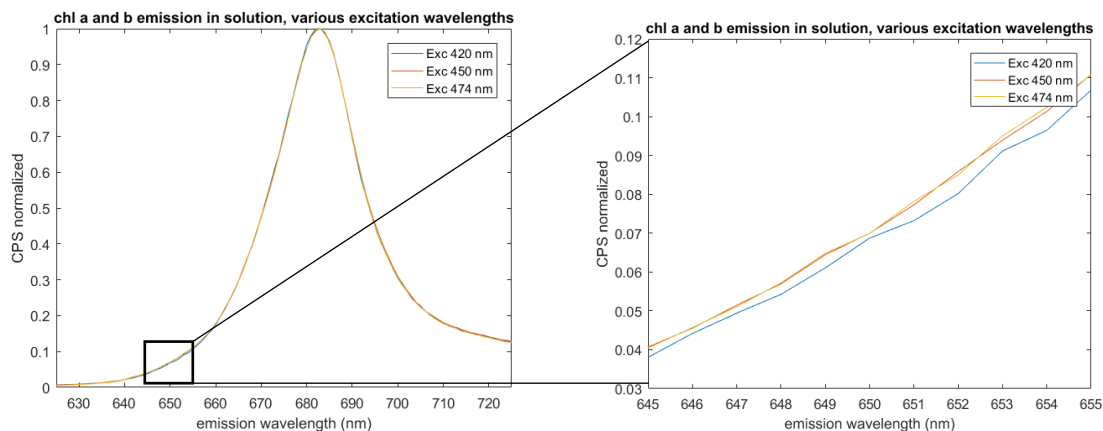


Figure 4.2: chl a and b in ethanol solution. The fluorescence emission was monitored across wavelengths from 630 nm to 720 nm, exciting at 420 nm to excited the solet band of chl a, and 474 nm to excite the solet band of chl b. The third excitation wavelength of 450 nm was tested as well and demonstrated that a significant portion of chl b molecules are excited at 450 nm.

In *wt* plants, when chl *b* was excited to preferentially excite LHCII, the lifetime was 210 ps longer at a detection wavelength of 680 nm and 180 ps longer at a detection wavelength of 690 nm, as shown in the table below (table 4.2). The decays measured at 420 and 450 nm excitation wavelengths and detected at 680 nm are shown in Fig. 4.3.

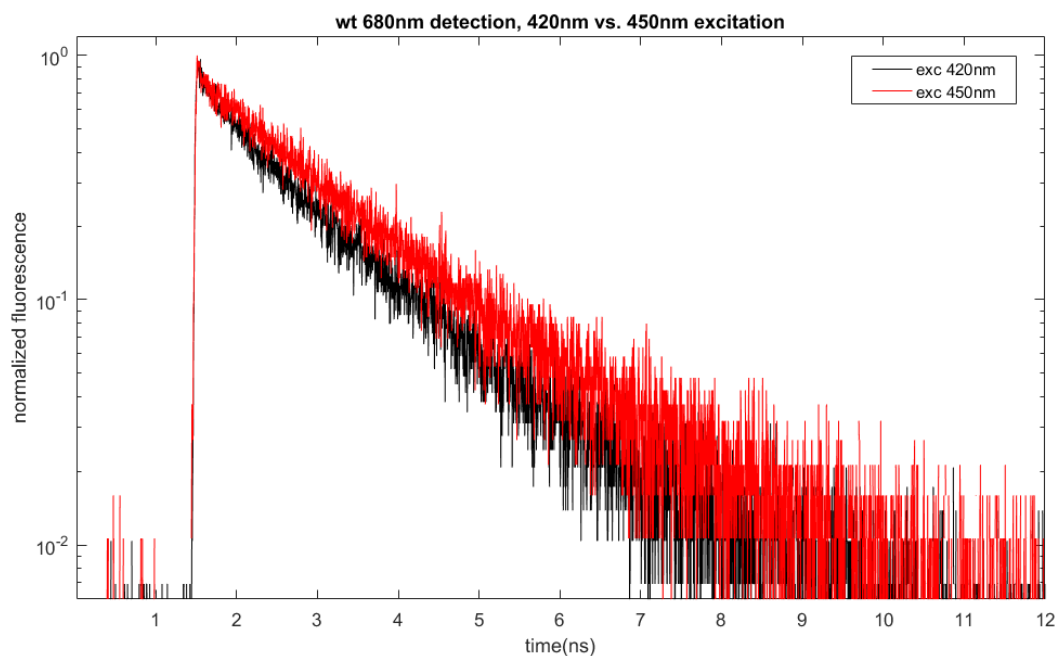


Figure 4.3: Fluorescence lifetime decays of *wt* plants excited at 420 vs. 450 nm and detected at 680 nm.

Detection wavelength	Excitation wavelength		Measured difference in τ_{avg}
	420 nm	447 nm	
	680 nm	690 nm	
	$\tau_{\text{avg}} = 1.05 \pm 0.06 \text{ ns}$	$\tau_{\text{avg}} = 1.26 \pm 0.12 \text{ ns}$	$0.210 \pm 0.134 \text{ ns}$
	$\tau_{\text{avg}} = 0.84 \pm 0.03 \text{ ns}$	$\tau_{\text{avg}} = 1.02 \pm 0.07 \text{ ns}$	$0.180 \pm 0.076 \text{ ns}$

Table 4.2: Measured average lifetime of wt plants for excitation wavelengths of 420 and 447 nm, and detection wavelengths of 680 and 690 nm. The errors shown are one standard deviation and the error on the difference is calculated by simple error propagation

However, when chl *b* was excited in *soq1 otk1* plants, there was no significant difference in lifetime at either 680 or 690 nm detection wavelengths (fig 4.4 and table 4.3).

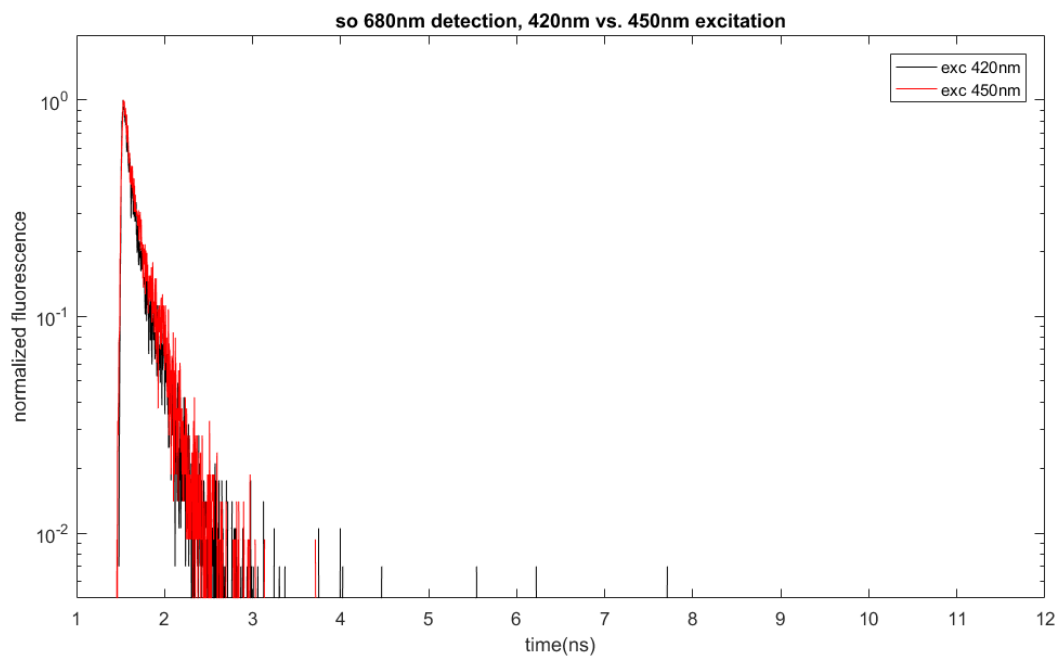


Figure 4.4: Fluorescence lifetime decays of *soq1 otk1* mutant excited at 420 and 450 nm and detected at 680 nm.

		Excitation wavelength		Measured difference in τ_{avg}
		420 nm	447 nm	
Detection wavelength	680 nm	$\tau_{avg} = 0.137 \pm 0.033$ ns	$\tau_{avg} = 0.132 \pm 0.021$ ns	0.005 ± 0.039 ns
	690 nm	$\tau_{avg} = 0.139 \pm 0.029$ ns	$\tau_{avg} = 0.130 \pm 0.019$ ns	0.009 ± 0.035 ns

Table 4.3: Measured average fluorescence lifetimes of *soq1 otk1* excited at 420 nm and 447 nm and detected at 680 and 690 nm.

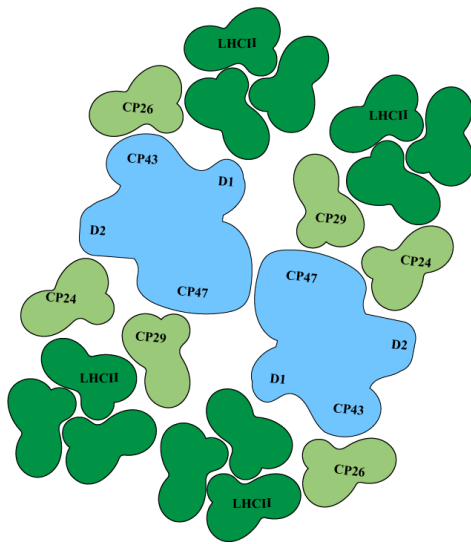


Figure 4.5: A cartoon of the PSII super complex

Discussion

A cartoon of the photosystem II (PSII) super complex is pictured to the left in Figure 4.5. The darkest green blobs arranged in groups of three represent the major light harvesting complexes of PSII, light harvesting complex II (LHCII), while the lighter green blobs represent the minor light harvesting complexes of PSII. The blue blobs are a representation of the core complexes including several pigment protein complexes, CP43 and CP47, and the reaction centers of PSII, D1 and D2. Whereas the core complexes have been shown to bind about 15 chl *a* molecules and no chl *b* [24], [25], CP26 and CP29 bind about 13 chlorophyll molecules each with a chl *a/b* ratio of about 2.5-3 [26], CP24 binds 10 chlorophylls at a chl *a/b* ratio of approximately one [27], and the LHCII monomers which make up the dark green colored LHCII

trimers pictured here, bind 13 or 14 chlorophylls with a chl *a/b* ratio of about 1.33 [28]. In 2010, van Oort et al. showed that isolated thylakoids exhibit a greater increase in fluorescence lifetime than BBY prepared membranes when excited at chl *b* rather than chl *a*. They attributed the difference to the increased chl *b* content of LHCII and the comparatively larger number of LHCII trimers at longer distances from PSII reaction centers and with fewer energetic connections to them in thylakoids. The migration time was calculated from the measured average lifetime and the modeled trapping time to be 150 ps in thylakoids [27] and 35 ps in BBY prepared membranes [28]. Van Oort et al. concluded that the migration time of excitations from antenna to PSII reaction centers is four times slower in thylakoids than in BBY prepared membranes due to additional LHCII complexes in thylakoids relative to the BBY membranes, which only contained C2S2M2 PSII super complexes like the one pictured above [27]. *In vivo*, as well as in intact thylakoids, there are LHCII trimers unassociated with any particular PSII reaction center. These LHCII complexes are less energetically connected to PSII complexes by definition but also may be significantly farther from any reaction center than other antenna complexes. Because of the distribution of chl *b* in the pigment protein complexes (PPCs) and the differences in absorption wavelength in the Soret bands of chl *a* and *b*, it is possible to preferentially excite LHCII trimers. In wild type plants, when chl *b* was excited the lifetime was 210 +/- 134 ps longer than when chl *a* was excited, in agreement with assertions made by van Oort et al. in 2010 regarding thylakoid membranes vs. BBY membranes.

Using a coarse-grained model described by Broess et. al [31] in which all excitation originates on chl *a* molecules and the rate of energy transfer between PPCs is determined by their connectivity to one another and their relative chl *a* contents, the excitation migration in PSII was modeled and the calculated average fluorescence lifetime determined to be 151ps. The model was then modified such that the excitation originated on chl *b* molecules only (energy transfer was still modeled by chl *a* content as in the case when chl *a* was the origin of excitation) and the calculated average lifetime increased to 180 ps. If the 29 ps difference between the modeled average fluorescence lifetime when chl *b* is excited rather than chl *a*, is multiplied by four to account for the fact that there are additional free LHCII trimers in the whole organism relative to the model, a change in migration time of 116 ps is obtained. Table 4.2 shows differences in wt

plant fluorescence lifetime calculated from the measured lifetimes and their standard deviations (from 10 samples) for detection wavelengths of 680 and 690 nm. The difference in lifetimes collected at 680 nm when excited at 420 vs 450 nm is 210 ± 134 ps while that detected at 690 nm is 180 ± 76 ps. The theoretically calculated difference in migration time of 116 ps falls within the error of the measured differences indicating that the change is reasonably attributable to the increased migration time when excitation originates on chl *b*, and thus on LHCII complexes, as opposed to chl *a*.

Pigment analysis revealed that the *soq1 otk1* mutant contains higher levels of the antenna pigments chl *b* and neoxanthin, and lower levels of the reaction center pigments chl *a* and beta-carotene leading to the conclusion that the mutant has more LHCII trimers and fewer PSII reaction centers. Given the additional LHCII trimers and the results of van Oort et al. in 2010 demonstrating that when more (disconnected) LHCII trimers are present, the migration time, and thus the fluorescence lifetime is longer, we would expect to see an increase in lifetime in the mutant when exciting chl *b*, as we saw in *wt* plants. However, we see no significant change in the lifetime (Table 4.3 and Fig.4.4). When detecting at 680 nm, 420 nm excitation led to a measured average lifetime of 137 ± 33 ps while 447 nm excitation yielded an average lifetime of 132 ± 21 ps. When detecting at 690 nm, 420 nm excitation led to a measured average lifetime of 139 ± 29 ps while 447 nm excitation yielded an average lifetime of 130 ± 19 ps. Each of these values is within one standard deviation of the others and as such, the values are statistically indistinguishable.

Upon treatment with DCMU, a chemical used to reduce the electron carrier, quinone A in order to chemically close PSII reaction centers, the *soq1 otk1* mutant exhibited a minimal increase in fluorescence yield with a slow rise time compared to *wt* plants (measurements done by Cynthia Amstutz and Alizée Malnoë in Niyogi lab). Because fluorescence rise time in the presence of DCMU is an inverse indicator of the functional antenna size, the slow rise of fluorescence suggests a small functional antenna size in the *soq1 otk1* mutant. In summary, the *soq1 otk1* mutant has more LHCII trimers as revealed by pigment analysis, but treatment with DCMU indicated that these antenna complexes are not energetically connected to PSII. If the LHCII trimers were not quenched, we would expect the increased number of LHCII trimers and the absence of energy transfer to reaction centers to lead to longer lifetimes when LHCII is preferentially excited. However, the fluorescence lifetime data shows no significant difference in the fluorescence lifetime when chl *b* is excited rather than chl *a*, suggesting that the LHCII trimers are strongly quenched in the mutant.

Conclusion

In conclusion, it appears that the mutant has additional LHCII trimers that are likely strongly quenched and possibly spatially separated from PSII reaction centers. The fluorescence lifetimes of aggregated LHCII trimers and monomers were measured to be 191 and 101 ps respectively [32]. The measured lifetime of 135 ps in the mutants regardless of excitation and detection wavelength likely reflects the short lifetime of LHCII aggregates that are very strongly quenched, thereby rendering diffusion to the reaction centers undetectable. It seems likely that we have measured the lifetime of aggregated LHCII *in vivo*. Whether or not the LHCII trimers in *soq1* are spatially separated from PSII reaction centers is unclear because a scenario could also be imagined in which the strongly quenched LHCII trimers are interspersed among the other

PPCs, leading to a very quenched lifetime regardless of where the excitation originates (on minor or major LHCs or even in the reaction centers).

Supplementary info/figures:

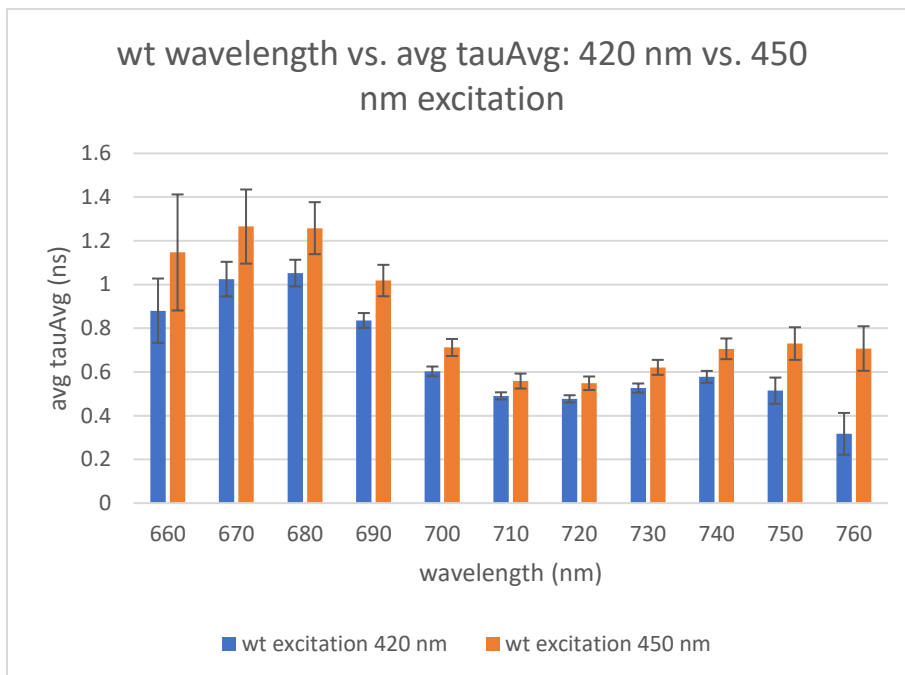
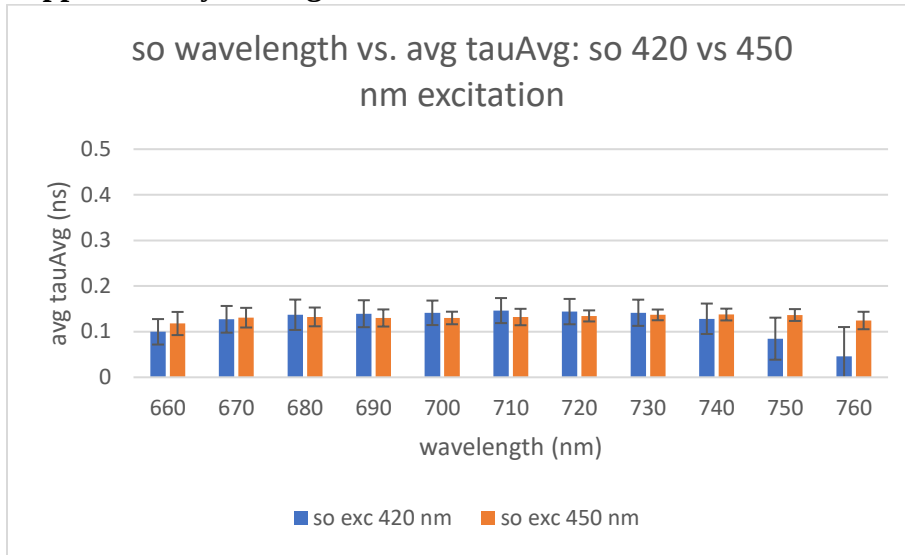


Figure S1: Average lifetimes measured at 420 and 450 nm excitation wavelengths and detected across a range of wavelengths from 660 – 760 nm. The error bars represent one standard deviation from the mean as calculated from the 10 samples. The lack of variation across wavelengths in the *soq1 otk1* mutant indicates that the quenching in LHCII trimers occurs at such a rate that it masks detection of exciton diffusion to the reaction centers of the mutant.

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Chapter 5: Development of Data Analysis Methods for TCSPC Snapshots

Introduction

The TCSPC snapshot method was developed in the Fleming lab by Amarnath et al. in order to follow light adaptation in *Chlamydomonas reinhardtii* via fluorescence lifetime measurements to better understand nonphotochemical quenching, or NPQ [1]. The apparatus was later adapted by Sylak-Glassman et al. to take similar measurements on whole leaves of *Arabidopsis thaliana* during light acclimation [2]. Both experiments were designed after traditional pulse amplitude modulated fluorescence yield measurements, or PAM traces, which allow for measurement of fluorescence yield from whole leaves with PSII reaction centers effectively closed by a saturating pulse, during light acclimation in order to measure parameters related to NPQ (see review by Matthew Brooks et al. for further details on PAM traces [3]). Compared to PAM traces, the TCSPC snapshot method adds value because it reveals additional, time-resolved information relative to fluorescence yield measurements, and it is insensitive to confounding factors that can influence yield measurement such as chloroplast migration and damage to fluorophores. However, the method also presented new challenges in the form of managing very large data sets, and balancing needs, such as short laser exposure time to avoid inducing undesirable processes, with other requirements like obtaining enough counts to achieve statistical significance.

The data consists of a series of snapshots taken during a sequence of light conditions designed for a particular experiment. Each snapshot consists of a one second period during which the sample is exposed to the laser and fluorescence is detected. Each snapshot is further divided into 0.2 second steps, and the step with the longest lifetime will later be chosen to obtain a measurement when reaction centers are closed to saturation, in a procedure similar to methods used in analysis of PAM data. Traditionally, snapshot data obtained via the TCSPC snapshot method have been aligned according to their maxima, and summed to obtain an effective average. Once the data were summed, the step with the longest lifetime was chosen as the step with reaction centers closed and the rest of the data were discarded. There were several problems with this method of data analysis. First, even within a single leaf from a single plant, the laser exposure time required to close reaction centers to saturation varies and as a result, summing the decays before choosing the step with reaction centers closed fails to ensure that the reaction centers were in fact closed for each individual measurement. However, if each individual curve is to be fitted before choosing the step with the reaction center closed, the dataset quickly becomes prohibitively large when using software to analyze the data manually. Secondly, summing the data does not appear to give the same result as fitting the curves individually and reconstructing the curve. In fact, summing introduces the need for a third, physically meaningless component as is evidenced by the ability to fit individual curves with two components easily (Fig. 5.1). Moreover, aligning according to the maxima of the decays appears to worsen this problem (Fig. 5.2 and 5.3). Singular value decomposition analysis, or SVD also confirmed that two components are sufficient in representing the majority of the variation in the data (Fig. 5.4).

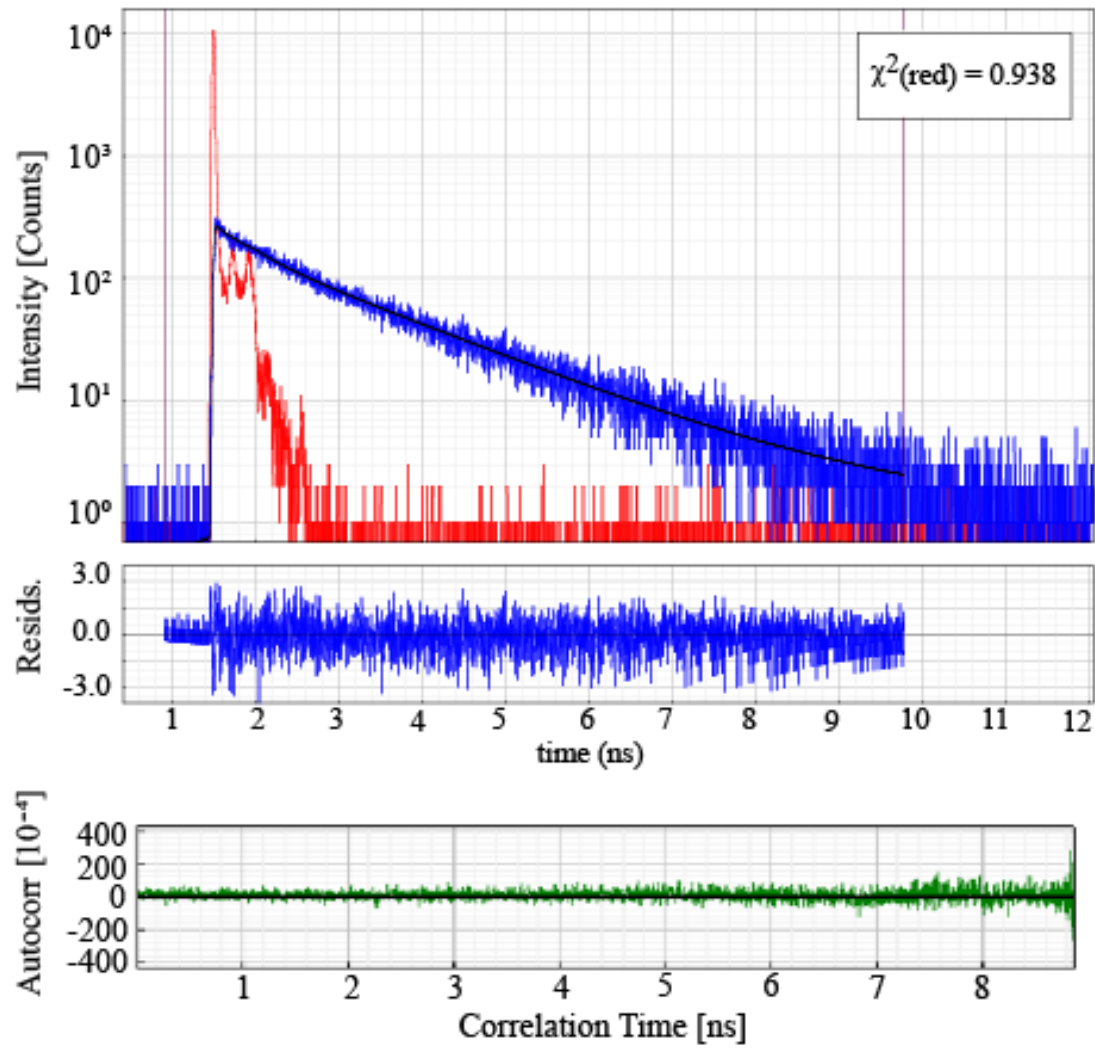


Figure 5.1: A single fluorescence lifetime decay of a wt leaf, dark adapted for 30 minutes. The measurement was taken in darkness and the data fitted to a bi-exponential function. It is clear from the residuals and the autocorrelation function that two components are sufficient to fit the data. The reduced chi squared value is 0.938.

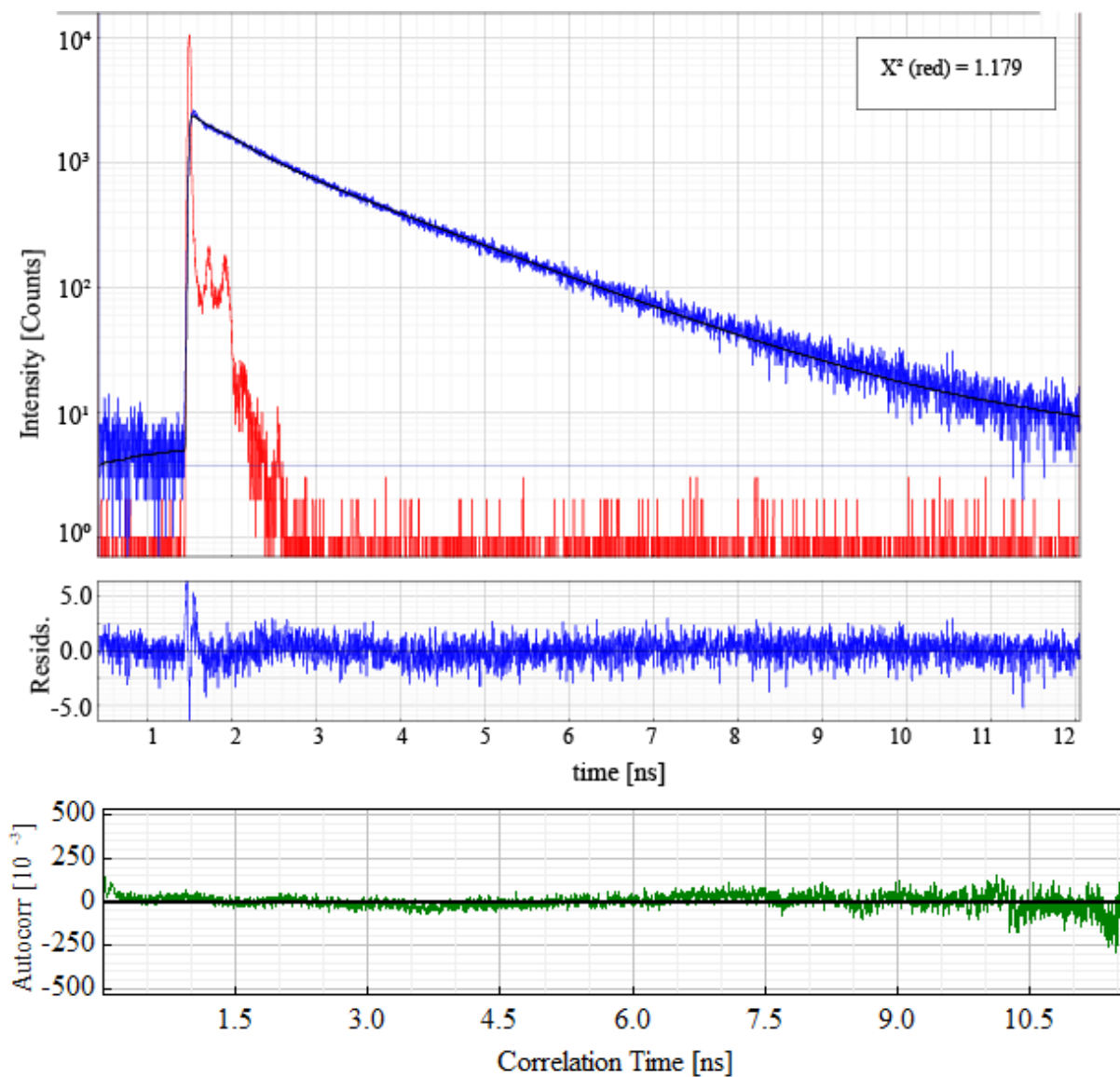


Figure 5.2: Upon summing the decays of the step chosen with the reaction center closed for 10 different leaves it is clear that the data can no longer be fitted to a bi-exponential function. The residuals have a clear pattern at early times, suggesting the data are not well aligned. The autocorrelation function also displays structure indicating a poor fit.

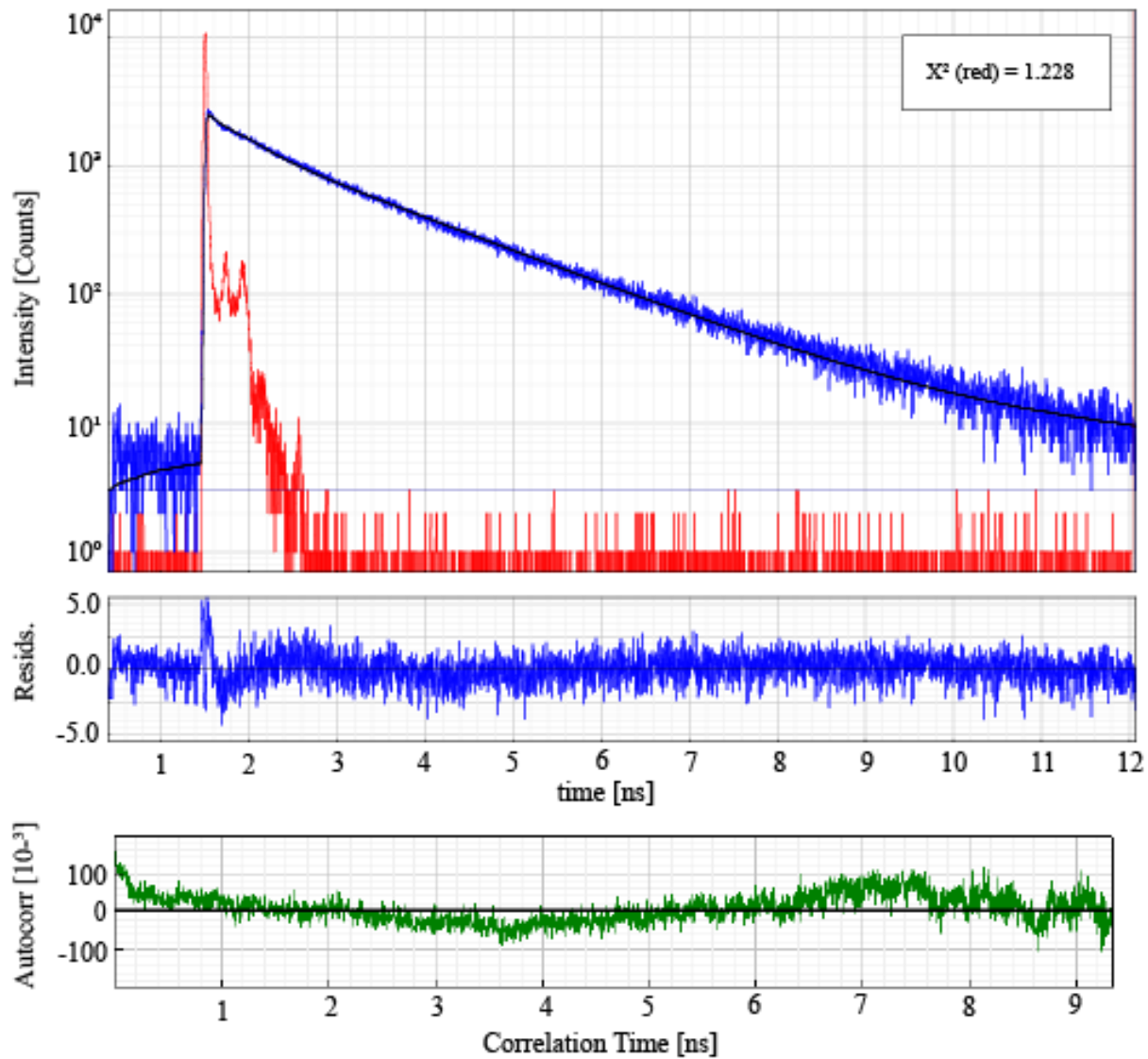


Figure 5.3: In an attempt to mitigate this problem, the data were first aligned according to their maxima and then summed. However, this made the fit worse than simply summing the data did, with an increased reduced chi squared value and increased structure in both the residuals and the autocorrelation of the residuals.

Singular Value Decomposition Results

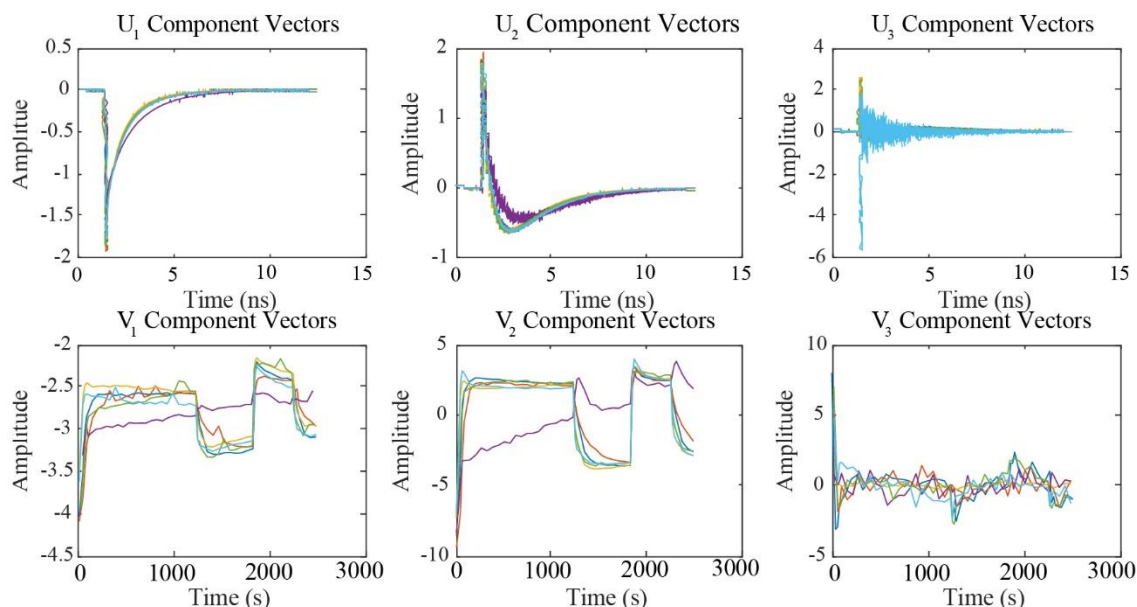


Figure 5.4 Singular Value Decomposition (SVD results for a large data set, consisting of wt plants as well as five other mutants. For further analysis of this data set, see chapter 4. The average U and V vectors corresponding to the largest three singular values are shown for wt (blue), lut2 (red), szl1 (yellow), szl1npq1+NoZEP1 (purple), szl1+NoZEP1 (green), and szl1npq1 (cyan). Using the standard definition of the SVD, $X = USV^*$, the decompositions were performed on matrices, X, composed of the vectors of raw fluorescence lifetime data corresponding to the actinic time points observed for a sample. The resulting decompositions were averaged over the 20 samples for each line. In this construction, U is composed of the actinic light acclimation basis vectors, V is composed of the ultrafast dynamics basis vectors, and S holds the singular values. The first two basis vectors of the ultrafast dynamics basis set clearly exhibit structure; the third exhibits far less structure. The actinic light acclimation basis set vectors exhibit two components with structure, the third exhibits noise. The singular values corresponding to the sets $\{U_1, V_1\}$, $\{U_2, V_2\}$, and $\{U_3, V_3\}$ drop by an order of magnitude between each set. This indicates that fitting more than two components does not extract additional meaningful information.

Singular Value Decomposition, or SVD, confirms the validity of bi-exponential non-linear least squares, or NLLS fits. The singular value decomposition is an analysis that resembles an eigendecomposition of a matrix that is not diagonalizable, or a change of basis, if you will.

- $X_{mn} = U_{mm} S_{mn} V_{nn}^T$
 - X = matrix of raw data organized strategically
 - U = columns are the left singular vectors
 - V = columns are the right singular vectors
 - S = singular values along diagonal

The SVD algorithm reduces the data into the minimum number of orthonormal basis vectors along both, the kinetic (qE) time axis and the TCSPC time axis [4]. In this way, we take a high dimensional data set with significant variability and reduce it to a lower dimensional space that exposes the substructure of the original data more clearly and orders it from most variation to least. The data is organized with the raw fluorescence lifetime data (fluorescence counts) in 50 columns corresponding to the 50 snapshots taken during light acclimation (Fig. 5.5).

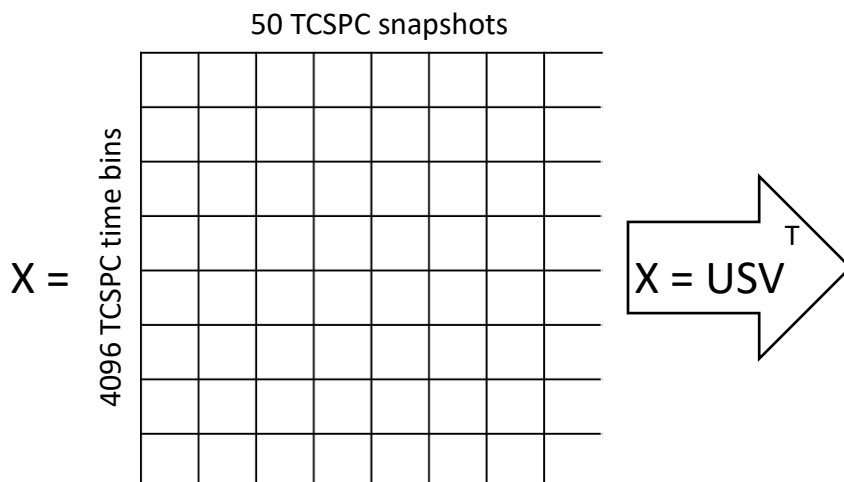


Figure 5.5: Organization of data for SVD analysis

The results of the SVD are interpreted as follows: The columns of U are the qE basis vectors, the columns of V are the TCSPC axis basis vectors, and S holds singular values which are essentially scaling factors. As can be seen in Fig. 5.4, the first two basis vectors of the TCSPC axis basis set clearly have structure. The third appears to be structure-less the majority of the time but does exhibit some minor structural components in some samples, while other samples exhibit little to no structure at all. Since the TCSPC axis basis vectors strongly suggest that the data can be understood as a two component process, we look to the qE axis basis sets to confirm this. The first two basis set vectors, V_1 and V_2 exhibit clear structure that replicates the general shape of a TCSPC snapshot trace but the third is clearly noise, validating our use of a bi-exponential fit when analyzing the fluorescence lifetime decays.

Dealing with low photon counts and big data

Until recently it was thought that 10,000 fluorescence counts were needed for the results of the fit to be considered statistically significant but the limitations of the setup and of the experiment itself prohibit the accumulation of such a large number of counts in a single measurement. In the TCSPC snapshot setup the laser acts as both a saturating pulse and a measuring light, which means the sample must not be exposed to the laser long enough to induce NPQ processes because we intend to measure the response of the leaf to actinic light that mimics the sun rather than to the blue laser light. This, combined with the limitations of the detector and the potential to damage the sample at higher excitation energies, limits the counts in the maximum bin of fluorescence decays measured by our setup to about 500 counts. Because we were unable to obtain a measurement with enough counts, the data was summed in the original analysis procedure which posed problems described earlier in this chapter. However, recently Santra et al. demonstrated that for 200 total counts and greater, the maximum likelihood estimator, or MLE, always provides a standard deviation of less than 10% of the mean lifetime, and even at 20 total counts there is only 20% error in the mean lifetime [5]. This new development allows for fitting fluorescence lifetime data with fewer counts in the maximum bin and therefore creates the opportunity to fit each fluorescence lifetime snapshot individually thereby eliminating the need for the third, physically meaningless component, and allowing the step with the reaction center closed to be chosen for each individual sample rather than averaging across all samples. The

remaining challenge arises because there are simply too many decays to fit manually in a reasonable amount of time. For example, if an experiment consists of 20 snapshots, each made up of 5 steps, and there are 3 types of plant sample to measure, and 20 leaves per sample type, then the total number of decays in the data set will be 6000 curves. In order to mitigate the time consumption and tedium of data analysis, I designed a series of Matlab functions to automate many steps of the data analysis. The original fit function was written by Kapil Amarnath and Sam Park in the Fleming lab and was modeled after Picoquant's Fluofit exponential reconvolution fitting procedure. The statistical analysis was guided by Jonathan Morris in the same lab.

Code Description

In order to avoid the need for additional, physically meaningless lifetime components each curve was fitted individually to a biexponential function using non-linear least squares analysis rather than aligning the curves according to their maxima and summing them to average the data before fitting. This method also allowed for the step with the reaction center closed to be chosen before any averaging was done ensuring that the reaction centers were closed in each leaf for each snapshot rather than just on average. Standard deviations on each fit parameter were calculated from the Jacobian and a reduced χ^2 value was calculated for each fit in order to confirm goodness of fit. Residuals of several curves from each dataset were examined as an additional check on goodness of fit. Furthermore, singular value decomposition revealed only two components, validating the use of a bi-exponential function to fit the data (Fig. 5.4).

Once each curve was fitted to a bi-exponential decay function, the average lifetime associated with each decay was calculated and the uncertainty associated with it determined from the standard deviation of the fit parameters. Next, the step with the longest lifetime was chosen as the step with the reaction centers closed. At this point, the rest of the data was discarded and only the data collected when the reaction centers identified as closed was further analyzed. The fits for each snapshot were used to calculate uncertainty-weighted averages of each of the components across 20 leaf samples. Due to normal variability in fluorescence yield from different leaves it was necessary to normalize the amplitudes associated with the decay times to sum to one in order to make them comparable across all samples. The average amplitude weighted lifetime across 20 samples was calculated for each snapshot from the uncertainty weighted averages of the fit parameters and the uncertainties on the parameters were then used to calculate the uncertainty on the average amplitude weighted lifetime for each decay. Each decay time, amplitude and average amplitude weighted lifetime for each snapshot/decay was then bootstrapped by examining the variation across resampling of the data from the 20 samples collected for each snapshot during TCSPC. Since the amplitudes were normalized to sum to one, their uncertainties cannot be decoupled. The uncertainty on the amplitude of the shorter decay time was calculated first, and the uncertainty on the amplitude of the larger decay time was then back calculated. The standard deviations were obtained by calculating a 68% confidence interval from the resampled dataset generated during bootstrapping. These confidence intervals were used to generate error bars on the traces of amplitude weighted average lifetimes during TCSPC measurements.

Code

FitFramework.m

This is the main function of the data analysis code. The data is assumed to be fluorescence lifetime snapshot data obtained with the Fleming lab's TCSPC setup. The raw data files from the Becker and Hickl data acquisition software are converted from .sdt files to .asc files for processing. For best results, the data should be organized as follows:

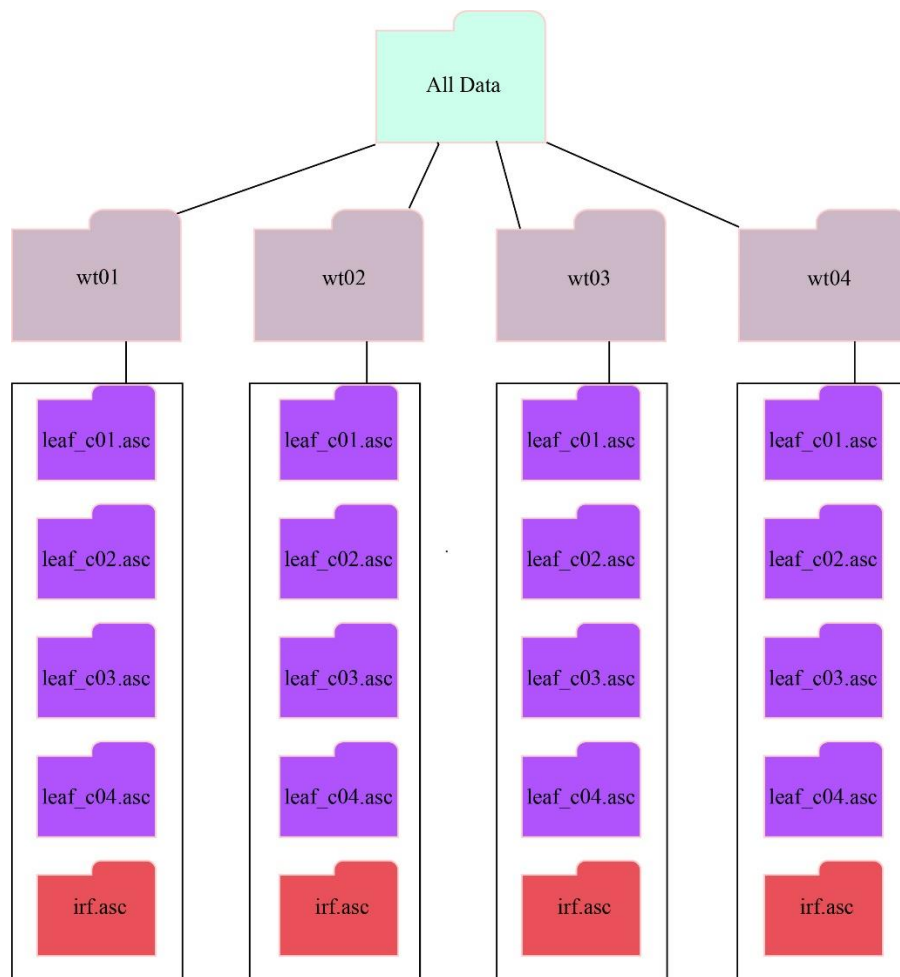


Figure 5.6: When using the fitting code described below, organize the data as picture above. Be careful to include zeros for numbers less than ten so that the MatLab list function lists the data in an intuitive manner. Convert all data and irf files to ascii files using the Becker and Hickl data acquisition software before beginning. An irf data file should be include in each sample file along with the snapshot files for that sample.

FitFramework.m is the main function that loads the raw data and irfs, makes an initial guess at fitting the very first decay using the method of curve stripping (**cite**). Once the first decay is fitted, the parameters that best fit that data are employed as the initial guess for the following curve. Because during a typical TCSPC snapshot experiment the decays change gradually over time, the fit of the previous decay should be an appropriate guess for the decay that follows.

The input parameters are as follows:

1. parent = the folder containing all of the data to be analyzed for a particular sample type (labeled 'All Data' in the folder structure of figure 5).
2. distinguish = the part of the file name for a particular sample type that is the same for all files in that sample type folder. In figure 5, distinguish is 'wt'.
3. Taxis = is the TCSPC time axis and usually ranges from 0-12.5 ns with a total of 4096 time bins.
4. nsteps = the number of steps each measurement is divided into in order to isolate a time period when reaction centers are closed to saturation. There are typically 5 steps per snapshot in our data.
5. numbins = the number of bins used to discretize the arrival time of the photons detected. Usually there are 4096 bins.
6. ncomp = the number of components to fit to. *NOTE this code is currently only functional with bi-exponential fits. The code will need to be altered to fit data to 3 components if desired but this should not be necessary as all mutants studied thus far appear to yield decays that can be fit to a bi-exponential function.

The outputs are as follows:

1. AllData is a four-dimensional array containing one dimension populated with the 16 parameters for each sample, snapshot and step. The array is sized according to (nleaves x nsnaps x nsteps x 16). The 16 fit parameters are described below in figure 6:

The 16 parameters describing the fits are stored in the fourth dimension of the AllData array

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A ₁	A ₂	τ_1	τ_2	bkgd shift	time shift	$\sigma(A_1)$	$\sigma(A_2)$	$\sigma(\tau_1)$	$\sigma(\tau_2)$	$\sigma(\text{bkgd shift})$	$\sigma(\text{time shift})$	τ avg	χ^2_{red}	χ^2	DOF

Figure 5.7: AllData is a 4-D array with the 4th dimension containing 16 parameters describing the bi-exponential fit to each raw decay. Size(AllData) is nleaves x nsnaps x nsteps x 16.

The FitFramework function depends on several other functions, the code for which can be found below along with descriptions of the inputs, outputs and what the code does.

FitFluoLifetimeNoPlot.m

This function was originally written by Kapil Amarnath and Sam Park in the Fleming lab at UC Berkeley and has been built upon to design the full data analysis code. The fitting procedure closely follows the exponential reconvolution method of non-linear least squares fitting used by picoquant Fluofit software [6].

The inputs to the fit function are as follows:

1. Taxis = is the TCSPC time axis and usually ranges from 0-12.5 ns with a total of 4096 time bins.
2. irf = a vector holding the instrument response function
3. data = a vector holding the raw fluorescence count data
4. numbins = the number of bins used to discretize the arrival time of the photons detected. Usually there are 4096 bins.

5. ncomp = the number of components to fit to. *NOTE this code is currently only functional with bi-exponential fits. The code will need to be altered to fit data to 3 components if desired but this should not be necessary as all mutants studied thus far appear to yield decays that can be fit to a bi-exponential function.
6. k0 = a vector holding the initial guess parameters. This will be the starting point of the search for a minimized chi squared value.
7. lb = lower bound of the parameters listed in k0
8. ub = upper bound of the parameters listed in k0

The outputs of the function are as follows:

1. tA_min = a vector holding the optimized parameters of the fit. The parameters appear in the same order they do in the fourth dimension of the AllData array above in figure 6.
2. chi2 = is the chi squared value associated with the fit
3. tauavg = the amplitude weighted average lifetime obtained from the fit in nanoseconds
4. tA_min_sig68 = a vector containing the standard deviations associated with each of the parameters listed in tA_min, in the same order. These sigmas are calculated from the Jacobian.
5. chi2red = the reduced chi squared value, calculated by dividing the chi squared value by the degrees of freedom associated with the measurement.
6. DOF = degrees of freedom associated with the measurement.

PlotFitTCSPCNoPlot.m

The original function produced a plot but this was unnecessary for our purposes so that portion of the original code was removed. The function now simply returns the calculated amplitude weighted average lifetime and the chi squared value associated with it. The code was initially written by Kapil Amarnath.

CalcError.m

This code calculates the error between the fit of the data and the actual data. The input parameters are as follows:

1. tA = the fit components listed in the first 6 positions of the fourth dimension of the AllData array described in figure 6.
2. Taxis = the time axis associated with the decays
3. irf = instrument response function (a vector)
4. data = a vector containing the raw fluorescence data
5. weights = to properly weight the residuals. See fluofit manual, p. 25 [6]
6. ncomp = number of fit components. Usually 2. Recall, the FitFramework.m code must be modified to fit the data to anything other than a bi-exponential function.
7. range = the range of data to be fitted as determined by the **FindRange.m** function described next.

FindRange.m

Finds the appropriate range of data to fit. The only input is the data array and the only output is the range that is found. This code was written by Kapil Amarnath and modified by myself.

Convolve.m

This function convolves the guess function with the instrument response function before comparing it to the raw data and calculating errors.

InitialGuessFinder.m

This function uses the curve stripping method to find an initial guess of the parameters to be used as input for k0 in **FitFramework.m**. The function determines the bin range, scans through a range of start bins to fit the long lifetime component of the first snapshot to determine the point at which the error is minimized by minimizing chi squared. This process gives a good starting guess for the longer lifetime component. The amplitude is then found by extrapolating the long lifetime curve back to the y-intercept. The shorter lifetime component is found by feeding guesses for the amplitude and lifetime into the **FitFluoLifetimeNoPlot.m** fit code from a grid. This gets us in the right neighborhood. The k0 is the output of this function and can be fed into **FitFluoLifetimeNoPlot.m**. The inputs to the function are the irf, the data, numbins and ncomp. The format of these inputs are the same as they were in previous functions and the only output is the k0 vector to be used in **FitFramework.m**.

DataPostProcess2comp.m

This function does error analysis on fits with 2 components. It takes the AllData file created by **FitFramework.m**, picks out the step with reaction centers closed, stepRCC, for each snapshot, averages across nleaves (number of samples), then determines error on parameters via bootstrapping. This version of the code picks out the step with reaction centers closed before any averaging is done.

getstepsRCCv2.m

This function finds and pulls out the fit parameters and errors associated with the step in each snapshot with the longest lifetime and thus with the reaction centers closed to saturation. The input parameters include the AllData array generated by **FitFramework.m**, nsnap (the number of snapshots), sample (the sample number of interest), nsteps (number of steps per snapshot). The only output is stepsRCC, an array that contains the step found to have the longest average lifetime for each snapshot - i.e. the step with the reaction centers closed.

***See Appendix A for full Matlab code with comments.**

References

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- [2] E. J. Sylak-Glassman, J. Zaks, K. Amarnath, M. Leuenberger, and G. R. Fleming, “Characterizing non-photochemical quenching in leaves through fluorescence lifetime snapshots,” *Photosynth. Res.*, vol. 127, no. 1, pp. 69–76, 2016.
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- [6] U. Manual and T. Data, *FluoFit*. .

Chapter 6: Conclusions and Outlook

Conclusions

As described in the introduction in chapter 1, many mechanisms of NPQ have been proposed and most of them are not mutually exclusive. It seems likely that nature has equipped plants with multiple mechanisms of photoprotection that may operate together or as backup protection in the event that another mechanism has failed. In this thesis, three different types of quenching were discussed: Fluorescence lifetime measurements on LHCII embedded proteoliposomes with different lipid compositions, described in chapter 2, suggest lipid composition likely impacts and may regulate LHCII trimer conformation in thylakoid membranes, thereby inducing aggregation quenching. In chapter three, TCSPC snapshots on six genetic mutants of *Arabidopsis thaliana* reveal that lutein and zeaxanthin probably both contribute directly to quenching in wild type plants and that their combined impact is greater than the sum of their individual quenching power. In chapter 4, a novel type of severe constitutive quenching dependent on the protein SOQ1 is shown to likely occur in aggregated LHCII trimers. Most likely these, as well as other quenching mechanisms that remain to be identified, operate together in nature to protect plants from damage under high light conditions. However, the presence of several mechanisms operating together *in vivo* complicates the interpretation of experimental results and as such, it makes sense to isolate the different processes using genetic mutations as well as spectroscopic techniques. Accurate models serve as a framework to explore the interaction of the various NPQ mechanisms and will likely be instrumental in future work elucidating how the different mechanisms interact.

Lastly, in chapter 5, it was shown that aligning and summing TCSPC snapshot fluorescence decays introduces significant errors in the data and should therefore be avoided. Instead, each individual decay should be fitted using the maximum likelihood estimator chi squared calculation and a bi-exponential function should suffice. Matlab code for processing very large data sets is provided in order to avoid having to fit the data manually using available software, which can take many hours. Additionally, the use of singular value decomposition to analyze TCSPC snapshot data is introduced.

Outlook

Because zeaxanthin and PsbS have a strong impact on quenching, they were not present in the LHCII embedded proteoliposomes described in chapter 2 to ensure that the quenching observed was most likely attributable to LHCII conformational changes alone. In future experiments, it would be interesting to determine whether LHCII conformational quenching induced by lipid composition is amplified by zeaxanthin and/or PsbS by measuring the impact of lipid composition on PPCs that do contain these pigments. In chapter 3, a complex experiment with 6 mutants, two light adaptation periods, and multiple HPLC measurements was described. It's clear that the addition of a second light period was instrumental in revealing new information regarding the contribution of zeaxanthin, lutein and how their respective xanthophyll cycles contribute to and modulate quenching. This leads us to suggest that adding more periods of illumination with varied frequencies and durations might allow us to separate out quenching mechanisms that occur on different timescales. Combining this newly proposed varying frequency periodic illumination technique with decay associated spectra techniques used to separate out the contributions of PSI and PSII to room temperature fluorescence could allow for

a better understanding of how lutein and zeaxanthin quenching differ with respect to the two photosystems. The coarse-grained model described in chapter 3 may offer further insight as well and further development of accurate and detailed models will be important when trying to understand how the various NPQ mechanisms interact.

Appendix A: MatLab code for data analysis procedure described in chapter 5

FitFramework.m

%Title: TCSPC snapshots fit code (fits data from multiple runs, same genetic line)

%Author: Michelle Leuenberger

%Version 1, May 28 2015

%** This version allows you to change the number of leaves, number of steps and number of snapshots easily through inputs

%Description:

%=====

=====
%This code will accept a parent file path associated with the genetic line being processed (exa: wt), open each sample file (wt01, wt02, ... , wt20), load each sample file along with its corresponding irf file (inside each sample file), then run through a nested loop structure to fit all of the fluorescence lifetime decays in each of the sample folders, storing the data in a massive array for further processing.

%Input arguments:

%=====

=====
%parent = file path to parent file where all 20 samples for that genetic line are stored
%distinguish = the part of the file names for the 20 samples that is the same (for example, 'wt' if samples are labeled, 'wt01', 'wt02', etc)
%Taxis = the time axis for the snapshots (usually Taxis =[0:0.0030525030525:12.5], followed by Taxis = Taxis';
%nsteps = number of steps per snapshot (usually 5)
%numbins = number of bins in the measurements (usually 4096)
%ncomp = number of components to fit to
%code = file path to the code needed for fitting

%Function dependence:

%=====

=====
%FitfluolifetimeNoPlot.m

 %PlotFitTCSPCNoPlot.m

 %CalcError.m

 %Convolve.m

 %FindRange.m

%InitialGuessFinder.m

%This code deals with blank snapshots

```

function [AllData] =
FitFramework(parent,distinguish,Taxis,nsteps,numbins,ncomp,code,nleaves,nsnaps)

AllData = zeros(nleaves,nsnaps,nsteps,16); %this is the array where ALL of the data from a
given genetic line will be stored. AllData stores tA_min params for each step in all 20 samples

Taxis = Taxis'; %convert Taxis to a column vector for easier handling
%k0 = [200,100,1.11,0.23,0,-0.01]; %can manually set initial guess if you want
lb = [0,0,0.5,0.05,0,-0.5]; %make a guess at lower bound and upper bound on fit params
ub = [1000,1000,3,1,10,1];

%go to parent file
cd(parent);
list = ls(['*',distinguish,'*']); %list (m-by-n char array,m = # of filenames,n = # characters for
longest name)
n = size(list,1);%get m, the number of file folders (usually 20)
for i=1:n
    files{i} = list(i,:); %files = a vector that contains the string names of all of the sample files
end

%get into the first file folder, get irf, load data
temp = [parent,'\',files{1}];
cd(temp);
irf = importdata('irf.asc','\n',10);
irf = irf.data;
data = importdata('leaf_c01.asc','\n',10);
data = data.data;
data = data(1:4096);

cd(code); %go to filepath for code
[k0] = InitialGuessFinder(irf,data,numbins,ncomp);
k0i = k0;
%This is the massive loop that runs through all of the sample files (usually 20), each containing a
TCSPC snapshot run, loading the irf and the data, fitting it, and storing the results
cd(parent);
lb = [0,0,0.5,0.05,0,-0.5];
ub = [1000,1000,3,1,10,1];

for i = 1:n %index through sample files
    k0 = k0i;
    samplefile = files{i}; %samplefile will be the ith sample file (wti, where i=01,02,03...10, for
example)
    parent2 = [parent,'\',samplefile];
    cd(parent2); %enter the sample file we want to work with now (wti folder)

    clear irf; %make sure the irf from the previous sample is gone

```

```

    irf = importdata('irf.asc','n',10); %import the appropriate irf data skipping the first ten lines
(header)
    irf = irf.data; %isolate the numerical data

snapfiles = ls('*.asc'); % list all ascii files in the sample folder (irf in there too)
numfiles = size(snapfiles,1) - 1; %subtracting 1 to account for irf in there (it's not a data file)

for j=1:numfiles
    cd(parent2);
    datafile = snapfiles(j+1,:); %the irf should always be the first file... so we skip it
    dataSnap = importdata(datafile,'n',10); %import data skipping the first ten lines (header)
    dataSnap = dataSnap.data; %isolate the numerical data from the dataSnap structure

    if max(dataSnap)<50 %check for blank snapshot
        AllData(i,j,:,:) = NaN; %if dataSnap is a blank, fill respective AllData cells with NaN
        continue %and exit the loop
    end

    cd(code); %go back to code folder
    l = 0; %reset indices for fit loop
    k = 1;
    %This is the loop where all the action happens - fit the data in each step and store the results
in an array
    while l < nsteps;
        clear data;
        data = dataSnap(l*numbins+1:k*numbins);
        [ tA_min, tA_min_Sig68, chi2, chi2red, DOF, tauavg ] =
FitfluolifetimeNoPlot(Taxis,irf,data,numbins,ncomp,k0,lb,ub);
        for m = 1:6
            AllData(i,j,k,m) = tA_min(m); %stores the 6 fit params in a giant matrix for all 20
samples
        end
        clear m;
        for m = 7:12
            AllData(i,j,k,m) = tA_min_Sig68(m-6);
        end
        AllData(i,j,k,13)= tauavg;
        AllData(i,j,k,14)= chi2red;
        AllData(i,j,k,15) = chi2;
        AllData(i,j,k,16) = DOF;
        k0 = tA_min;
        l = l+1;
        k = k+1;
    end
end
cd(parent);

```

```
    save AllData.mat;  
end
```

FitFluoLifetimeNoPlot.m

% Title: Fits time-resolved fluorescence data acquired from TCSPC to multiple exponentials.

% Author: Kapil Amarnath

% Version: 2.0

% Date: September 20, 2012

% Modified: May 29 2015 by Michelle Leuenberger

% Description:

% =====

% This is code that will fit an experimental fluorescence decay from a TCSPC measurement to multiple exponentials.

% User Inputs:

% =====

% 1. Taxis <-- Time axis used in calculation

% 2. irf <-- irf data (array)

% 3. data <-- data to be fit (array)

% 4. numbins <-- number of bins

% 5. ncomp <-- number of components

% 6. k0 <-- Initial point of search (in sequence: a1,a2,t1,t2,bkgdshift,timeshift)

% 7. lb <-- Lower bound of possible values in k0

% 8. ub <-- Upper bound of possible values in k0

% Outputs:

% =====

% 1. tA_min <-- vector with fitted parameters [Units: Char]

% 2. chi2 <-- χ^2 value from fit [Units: number]

% 3. tauavg <-- amplitude-weighted average lifetime of fit [Units:
% nanoseconds]

% 4. tA_min_Sig68 <-- sigmas on params to get 68% CIs

% 5. chi2red <-- χ^2/DOF should be near 1

% 6. DOF <-- degrees of freedom

% Function Dependence:

% =====

% 1. FindRange.m

% 2. CalcError.m

% 3. PlotFitTCSPCNoPlot.m

function [tA_min, tA_min_Sig68, chi2, chi2red, DOF, tauavg] =
FitfluolifetimeNoPlot(Taxis,irf,data,numbins,ncomp,k0,lb,ub)

% Load values, get weights and range to fit

% =====

```

tA = k0;
weights = zeros(numbins,1);
for i=1:numbins
    if data(i) ~= 0
        weights(i) = 1 / sqrt(data(i));
    else
        weights(i) = 1;
    end
end

range = FindRange(data);

% =====
% ===== Optimize Parameters by Fit =====
% =====

mini = 100;
for i=-20:20

    newrange = range + i;
    binrange = newrange(1):newrange(2);
    if data(newrange(1)) < 21
        f = @(x)CalcError(x,Taxis,irf,data,weights,ncomp,newrange);
        [tA_new,resnorm,residual,exitflag,output,lambda,jacobian] =
lsqnonlin(f,tA,lb,ub,optimset('TolX',1e-10,'TolFun',1e-10));
        %tA_new = tA_new;
        Cens = tA_new;
        [ferr, curve] = CalcError(tA_new,Taxis,irf,data,weights,ncomp,newrange);
        chi2new = sum(ferr.^2);
        DOFnew = (length(binrange)-(2*ncomp+2)-1);
        chi2rednew = chi2new/DOFnew; %degrees of freedom = 2*ncomp+2=6 since we are fitting
6 parameters
        %ferr is the diff*weights
        if chi2rednew < mini
            mini = chi2rednew;
            tA_min = tA_new;
            chi2 = chi2new;
            DOF = DOFnew;
            chi2red = chi2rednew;
            range_min = newrange;
            resid = residual;
            J = jacobian;
            ci = nlparci(tA_new,resid,'jacobian',J,'alpha',0.33); %confidence intervals from residuals
and jacobian
            for w = 1:(ncomp*2+2)
                tA_min_Sig68(w) = (ci(w,2)-ci(w,1))/2; %calc sigmas from confidence intervals

```

```
        end
    end
end
end
```

```
[ferr, curve] = CalcError(tA_min,Taxis,irf,data,weights,ncomp,range_min);
[chi2red, tauavg] = PlotFitTCSPCNoPlot(tA_min, ferr, curve, Taxis, data, ncomp, range_min);

fclose('all');

end
```

PlotFitTCSPCNoPlot.m

```
% Title: Plots the results of a fit of TCSPC data
% Author: Kapil Amarnath
% Version: 1.0
% Date: September 21, 2012
% modified June 1 2015 by Michelle Leuenberger to omit plotting feature and just return output values.
```

```
% Description:
% =====
% Takes fit information and returns tauavg and chi squared
```

```
% User Inputs:
% =====
% 1. tAfit <-- vector containing the fitted parameters (in sequence:
%    a1,a2,a3,t1,t2,t3,bkgdshift,timeshift)
% 2. ferr <-- error of fit (curve-data) (vector) [Units: counts]
% 3. curve <-- fitted data (vector) [Units: counts]
% 4. data <-- data to be fit (vector) [Units: counts]
% 5. Taxis <-- time axis used in calculation (vector) [Units: ns]
% 6. ncomp <-- number of components
% 7. range <-- range of bins used [startbin endbin] [Units: bin number]
```

```
% Outputs:
% =====
% 1. chi2 <-- chi^2 from fit [Units: number]
% 2. tauavg <-- amplitude weighted lifetime of fit [Unit: ns]
```

```
function [chi2red, tauavg] = PlotFitTCSPCNoPlot(tAfit, ferr, curve, Taxis, data, ncomp, range)
```

```
% =====
%chi2 = round(chi2*100)/100;
% Initiate Everything With Range
% =====
binrange = range(1):range(2);
proptaxis = Taxis(binrange);
chi2red = sum(ferr.^2) / (length(binrange)-(2*ncomp+2)-1);
% Calculate amplitude-weighted lifetime
% =====
amps = tAfit(1:ncomp)/(sum(tAfit(1:ncomp)));
taus = tAfit(ncomp+1:ncomp*2);
tauavg = dot(amps,taus);
tauavg = round(tauavg*100)/100;
```

```
end
```

CalcError.m

% Title: Calculates the error of a multi-exponential fit

% Author: Kapil Amarnath

% Version: 2.0

% Date: September 20, 2012

% Description:

% =====

% Calculates the error between the convolution of the sum of exponentials and the actual data.

% User Inputs:

% =====

% 1. tA <-- vector with fitted parameters (in sequence:

% a1,a2,a3,t1,t2,t3,bkgdshift,timeshift)

% 2. Taxis <-- Time axis used in calculation

% 3. irf <-- irf (array)

% 4. data <-- data to be fit (array)

% 5. weights <-- weights for properly weighting the residuals (See p. 25 of fluofit manual for
% more details.)

% 6. ncomp <-- number of components

% 7. range <-- range [startbin endbin] of fit

% Outputs:

% =====

% 1. FL_Err <-- vector with errors from subtracting fit from data [Units: counts]

% 2. curve <-- vector with curve generated from fit to data [Units: counts]

% Function Dependence:

% =====

% 1. Convolve.m

function [FL_Err,curve] = CalcError(tA, Taxis, irf, data, weights, ncomp, range)

% Extract parameters from tA vector

% =====

Amp = tA(1:ncomp);

tau = tA(ncomp+1:2*ncomp);

k = -1./tau;

bkgd = tA(length(tA)-1);

timeshift = tA(length(tA));

% Shift IRF

% =====

Taxisirf = Taxis(range(1):range(2));

newtp = Taxisirf + timeshift;

```

x = newtp; %make the shifted time axis the new time axis
y = irf(range(1):range(2));
xx = Taxisirf;
yy = pchip(x,y,xx); %find the values of irf at the old (xx) timepoints,
% given that the irf has shifted by timeshift
irfnew = yy;

irfnew = irfnew + bkgd;

% Calculate Curve Based on Fit
% =====
Fl = 0*Taxis;

for i=1:ncomp
    Fl = Fl+Amp(i)*exp(k(i)*Taxis);
end

curve = Convolve(irfnew,Fl);
curve = curve(:);

% Subtract Actual Data Curve from Calculated Curve to Get Error
% =====
data = data(:);
data = data(range(1):range(2));
diff = curve - data;
FL_Err = diff .* weights(range(1):range(2));

end

```

FindRange.m

% Title: Obtains a starting point/endpoint for a fluorescence decay fit.

% Author: Kapil Amarnath

% Version: 1.0

% Date: September 20, 2012

% Description:

% =====

% This code will obtain a [startbin endbin] range for
% fitting fluorescence decays.

% User Inputs:

% =====

% 1. data <-- full TCSPC data from one measurement [Units: counts]

% Outputs:

% =====

% 1. range <-- [startbin endbin] [Units: Int]

function [range] = FindRange(data)

% Find startbin

% =====

smoothdiffs = smooth(diff(data));

abovethirty = find(smoothdiffs > 10); %was set to 10 previously. Changed it to fit normalized
sums of decays (max diffs much smaller)

if numel(abovethirty)==0

 abovethirty = find(smoothdiffs> 5);

end

if numel(abovethirty)==0

 abovethirty = find(smoothdiffs>2.5);

end

if numel(abovethirty)==0

 range = 1;

 return

end

startbin = abovethirty(1);

% Find endbin

% =====

background = 2*mean(data(1:startbin));

if background < 4 %this threshold may need to be altered at times

 background = 4;

end

endbin = 3300;

```
data = smooth(data);  
while data(endbin) < background  
    endbin = endbin - 1;  
end  
  
% Set range  
% =====  
range = [startbin endbin];  
end
```

Convolve.m

```
function y = Convolve(irf, x)
% **Original code most likely written by Kapil Amarnath (unlabeled)
% This function convolves the irf with the data from the sum of exponentials, x.
% modified code to normalize and un-normalize - trying to get meaningful
% amps - 05/29/15 – modified by Michelle Leuenberger

% as of 06/01/2015 the modification works, producing Amps that are in number of counts
relative to total counts

irf = irf(:)';
irfNorm = sum(irf); %added normalization procedure
irf = irf/irfNorm;

x = x(:)';
xNorm = sum(x); %again, added normalization procedure
x = x/xNorm;

p = length(x);
n = length(irf);
if p>n
    irf = [irf zeros(1,p-n)];
else
    irf = irf(1:p);
end
y = real(ifft(fft(irf).*fft(x))); %convolution theorem
t = rem(rem(0:n-1,p)+p,p)+1;
y = y(t);
y = y*xNorm; %"Un-normalization" procedure

end
```

InitialGuessFinder.m

% Title: Obtains k0 vector input for FitFramework.m by curve stripping to find long lifetime
% params and then using a grid of guesses for the short lifetime component.

% Author: Michelle Leuenberger

% Version: 3.0

% Date: May 20, 2015

% Description:

% =====

% Determines endbin, scans through a range of start bins to fit the long lifetime component of
snapshot #1 determining the point at which the error is minimized (finding the min Chi2). Gives
a good starting guess for the longer lifetime component. Amplitude is found by extrapolating the
long lifetime curve back to the y-intercept (Amp). The shorter lifetime component is found by
feeding guesses for the Amp and lifetime into Kapil's fit code from a grid. This should get us in
the right neighborhood - the k0 returned can then be fed into Kapil's fit code for the next decay.

% User Inputs:

% =====

% 1.irf – instrument response function in a vector

% 2.data - TCSPC data from one measurement [Units: counts]

% 3.numbins – number of bins to discretize time (4096)

% 4.ncomp – number of fit components (likely 2)

% Outputs:

% =====

% 1. k0 <-- Initial guess values for the two lifetime components and their amplitudes

%function dependence:

%=====

%FitfluolifetimeNoPlot.m

function [k0] = InitialGuessFinder(irf,data,numbins,ncomp)

%define time axis:

Taxis = [0:0.0030525030525:12.5];

Taxis = Taxis';

dataRaw = data;

% Find startbin (directly from Fitfluolifetime.m code)

% =====

smoothdiffs = smooth(diff(data));

abovethirty = find(smoothdiffs > 30); %sometimes a value other than 30 is appropriate –

discover through trial and error

if numel(abovethirty) == 0

 abovethirty = find(smoothdiffs > 15);

if numel(abovethirty) == 0

```

        find(smoothdiffs>10);
    if numel(abovevethirty) == 0
        find(smoothdiffs>5);
    end
end
end
startbin = abovevethirty(1);

% Find endbin (directly from Fitfluolifetime.m code)
% =====
background = 2*mean(data(1:startbin));
if background < 4 %value may change – trial and error
    background = 4;
end
endbin = 3300;
data = smooth(data);
while data(endbin) < background
    endbin = endbin - 1;
end

data = dataRaw; %get back the unsmoothed data

% Get weights (directly from Fitfluolifetime.m code)
% =====
weights = zeros(numbins,1);
clear i;
for i=1:numbins
    if data(i) ~= 0
        weights(i) = 1 / sqrt(data(i));
    else
        weights(i) = 1;
    end
end
clear i;
% define new startbin to begin scanning for time point with lowest error for long lifetime
component
startbin = endbin - 600; %starting closer to endbin yielded very poor results
chi2reds = zeros;
j = 1;
clear M;
clear I;
[M,I] = max(irf);
t0 = Taxis(I);

while startbin > 656
    dataLintobe = data;

```

```

dataLintobe(dataLintobe == 0) = 2; %find and replace any 0s and 1s with 2s (ln(0) = -inf,
ln(1) = 0)
dataLin = log(dataLintobe(startbin:endbin,:)); %linearize the data in the range of interest
t = Taxis(startbin:endbin); % pick out the time points in that range from Taxis
p = polyfit(t,dataLin,1); %get the coefficients for the best fit line of the data
tauL = -1/(p(1)); % calculate the tau long (a guess for the long lifetime component)
lnA = p(1)*Taxis(I) + p(2); % calc ln(amplitude) at rise time of irf from equation of line
aL = exp(lnA); % calc Amp at time of max(irf)- this is the amplitude fluofit likes to calc
At0 = exp(p(2)); % This is the amplitude at t = 0 - used to generate Fl curve

Fl = 0*Taxis;
kL = p(1);
Fl = Fl + At0*exp(kL*Taxis); %curve for comparing to data and generating chi2 values

d = Fl(startbin:endbin) - data(startbin:endbin);
FL_Err = d .* weights(startbin:endbin);
chi2red = sum(FL_Err.^2) / (length(dataLin)-3); %3 because n=2 components to fit (/n-1)

chi2reds(j,1) = Taxis(startbin);
chi2reds(j,2) = startbin;
chi2reds(j,3) = chi2red;
chi2reds(j,4) = At0; %amplitude at time = 0ns
chi2reds(j,5) = aL; %amp at point where guess curve crosses rise of data/irf (time ~1.5ns)
chi2reds(j,6) = tauL;

j = j + 1; %index for error matrix
startbin = startbin - 10; % define next range to test
end

nIt = size(chi2reds,1);% # iterations in while loop above

minChi2red = 100;
%minChi2redbin = 1;
clear i;
for i = 1:nIt
    if chi2reds(i,3) < minChi2red
        minChi2red = chi2reds(i,3); %compare each chi2 to the previous chi2
        minChi2redbin = i; %if chi2 is smaller than previous chi2, replace minChi2 with that value
    end
end
clear i;

tauL = chi2reds(minChi2redbin,6);
aL = chi2reds(minChi2redbin,5); %amp at t0 for experiment (when irf arrives)
At0 = chi2reds(minChi2redbin,4); %amp for absolute t0

```

```

%have initial guess for long component - use tauL and aL to get guess for tauS and aS -->
%k0 = [aL,85,tauL,0.23,0,-0.01]; %setting initial guess to std guess with curve stripper vals for
long component
%ub = [(aL+10),500,(tauL+0.25),1,1,1]; %setting upper bound to limit long lifetime component
params
%lb = [(aL-10),0,(tauL-0.25),0.05,0,-0.5]; %setting lower bound to limit long lifetime
component params

guessGrid = zeros(2,11); %initialize guess grid
%fill guess grid with guess vals for short lifetime component params
lowAs = 25;
for GuGr = 1:11
    guessGrid(1,GuGr) = lowAs+10;
    lowAs = guessGrid(1,GuGr);
end
clear GuGr;

lowts = -0.03;
for GuGr = 1:11
    guessGrid(2,GuGr) = lowts+0.08;
    lowts = guessGrid(2,GuGr);
end
clear GuGr;

%set up array to store k0s and their respective chi2s
guesses = size(guessGrid,2);
k0s = zeros(guesses*guesses,7);
row = 1;
%run through each combination of AmpS/tauS in the grid, fit to get chi2, and k0 vector for each
trial. (use FitfluolifetimeNoPlot.m)
for AmpG = 1:guesses
    for tauG = 1:guesses
        k0 = [aL,guessGrid(1,AmpG),tauL,guessGrid(2,tauG),0,-0.01];
        ub = [(aL+100),(guessGrid(1,AmpG)+5),(tauL+0.4),(guessGrid(2,tauG)+0.04),10,1];
        lb = [(aL-100),(guessGrid(1,AmpG)-5),(tauL-0.4),(guessGrid(2,tauG)-0.04),0,-0.5];
        [ tA_min, tA_min_Sig68, chi2, chi2red, DOF, tauavg ] =
FitfluolifetimeNoPlot(Taxis,irf,data,numbins,ncomp,k0,lb,ub);
        chi2red = chi2red;
        k0s(row,1) = tA_min(1);
        k0s(row,2) = tA_min(2);
        k0s(row,3) = tA_min(3);
        k0s(row,4) = tA_min(4);
        k0s(row,5) = tA_min(5);
        k0s(row,6) = tA_min(6);
        k0s(row,7) = chi2red;
        row = row + 1;
    end
end

```

```
    end  
end  
  
clear M;  
clear I;  
[M,I] = min(k0s(:,7));  
k0 = k0s(I,1:6);  
end
```

DataPostProcess2comp.m

% Description: Error analysis with 2 components: Takes AllData file from FitFramework.m,
% picks out stepRCC for each snapshot, averages across nleaves (number of samples), then
% determines error on parameters via bootstrapping.

% Author: Michelle Leuenberger

% Version: 1.0

% Date: May 4th, 2016

%**NOTE this code only uses weighted averages (weighted according to the standard deviations
determined using the Jacobian, which works similarly to SPA using chi sqr ratios). ALSO: it
gets the steps with RCC before averaging!

%**NOTE the weighted averages of tauAvg are returned for AvgDataRCC while AllDataCIs
returns a regular old average.

%****REMEMBER getstepsRCCv2 assumes sample folders were labeled wt01, wt02, wt03...
and so on so that matlab's ls operator will list them intuitively, eliminating the necessity of a
Legend****

%Inputs →

%=====

% parent = file path to folder containing your AllData file generated by FitFramework.m for that
% particular genetic line

% code = file path to folder with code in it

% nsnaps = number of snapshots for this TCSPC run

% nsteps = number of steps in each snapshot (usually 5)

% nleaves = number of leaves (samples) I ran TCSPC snapshot measurement on for that genetic
% line - didn't call it nsamples because that is input for bootstrap.m function

% ncomp = number of fit components (for ease of writing code for dif ncomp)

% Outputs

%=====

% AllDataRCC -[nleaves,nsnaps,17] stepRCC is 17th param

% AvgDataRCC -[nsnaps,16] weighted averages of params for stepRCC of each snapshot

% AllDataCIs - holds confidence intervals determined by bootstrapping and assuming a gaussian
% distribution

% Fxn dependence:

%=====

% [bCI, samples] = bootstrap(data, nSamples)

% [stepsRCC] = getstepsRCCv2(AllData,nsnaps,sample,nsteps)

function [AllDataRCC,AvgDataRCC,AllDataCIs] =
DataPostProcess2comp(parent,code,nsnaps,nsteps,nleaves,ncomp)
cd(parent);

```

load('AllData.mat', '-mat', 'AllData'); %loads AllData array generated by FitFramework.m

%Normalize amps to sum to 1 before calculating tauAvgs bc amps are counts and fl yield varies
% strongly leaf-to-leaf. An average of amps in this form would be nonsense
%=====
AllDataAmps = AllData(:,:,1:ncomp);
SumAsAllData = sum(AllDataAmps,4);
clear f;
f = @rdivide;
FracAmps = bsxfun(f,AllDataAmps,SumAsAllData);
AllDataNew = AllData;
AllDataNew(:,:,1) = FracAmps(:,:,1);
AllDataNew(:,:,2) = FracAmps(:,:,2);

%Alter sigmas to reflect normalization:
sigALsqr = zeros(nleaves,nsnaps,nsteps,1);
sigAL = zeros(nleaves,nsnaps,nsteps,1);
sigALsqr = (((-AllData(:,:,1))./(AllData(:,:,1)+AllData(:,:,2)).^2)).^2).*((AllData(:,:,8)).^2)
+ ((1./(AllData(:,:,2)+AllData(:,:,1)) -
AllData(:,:,1))./(AllData(:,:,2)+AllData(:,:,1)).^2)).^2).*AllData(:,:,7).^2;
sigAL = sqrt(sigALsqr);
AllDataNew(1:nleaves,:(2*ncomp+3)) = sigAL;

sigASsqr = zeros(nleaves,nsnaps,nsteps,1);
sigAS = zeros(nleaves,nsnaps,nsteps,1);
sigASsqr = ((1./(AllData(:,:,2)+AllData(:,:,1))-
AllData(:,:,2))./(AllData(:,:,2)+AllData(:,:,1)).^2)).^2.*(AllData(:,:,8)).^2+((-
AllData(:,:,2))./(AllData(:,:,2)+AllData(:,:,1)).^2)).^2.*(AllData(:,:,7)).^2;
sigAS = sqrt(sigASsqr);
AllDataNew(1:nleaves,:(2*ncomp+4)) = sigAS;

AllDataNew(1:nleaves,:(ncomp+1):(2*ncomp+2)) =
AllData(1:nleaves,:(ncomp+1):(2*ncomp+2)); %fill in the rest of AllDataNew with values
%from AllData
AllDataNew(1:nleaves,:(3*ncomp+3):end) = AllData(1:nleaves,:(3*ncomp+3):end);

%find the steps with the longest average lifetime (rxn ctrs "closed")
cd(code);
clear i;
stepsRCCall=zeros(nleaves,nsnaps); %initialize array to hold stepsRCC for all leaves and snaps
for i = 1:nleaves %loop to get stepsRCC for all leaves and snaps
    [stepsRCC] = getstepsRCCv2(AllDataNew,nsnaps,i,nsteps,ncomp);
    stepsRCCall(i,:) = stepsRCC;
end

AllDataRCC = zeros(nleaves,nsnaps,1,(4*ncomp+9)); %initiate AllDataRCC

```

```

AllDataRCC(:,1,end) = stepsRCCall; %fill in parameter 17 with the stepsRCCallmatrix

%fill in the rest of AllDataRCC with the data for the appropriate step from AllDataNew.
clear leaf
clear snaps
for leaf = 1:nleaves
    for snap = 1:nsnaps
        AllDataRCC(leaf,snap,1,1:(end-1)) = AllDataNew(leaf,snap,stepsRCCall(leaf,snap),:);
    end
end

AllDataRCC = squeeze(AllDataRCC); %get rid extra dimensions

%getting weighted averages of all params:
%starting with aL
aLs = AllDataRCC(:,1); %separate aL param from the AllDataRCC for ease of matrix mult
% (bsxfun doesn't seem to work here)
wtaL = 1./(AllDataRCC(:,(2*ncomp+3)).^2); %weights = (1/(sigma(aL)^2)
wtaLxaL = wtaL.*aLs; %calc weights x aL (weighted values)
AvgDataRCC = zeros(nsnaps,(4*ncomp+2)); %initialize array to hold weighted averages of all
%params across 20 samples and their sigmas
AvgDataRCC(:,1) = (nansum(wtaLxaL,1)./nansum(wtaL,1)); %calc weighted averages for aL -
%you are summing the weighted values and dividing by the sum of the weights
AvgDataRCC(:,(2*ncomp+1)) = sqrt(1./(nansum(wtaL,1))); %calc the sigma associated with the
%weighted avg

%now aS
aSs = AllDataRCC(:,2); %pick out aS values
wtaS = 1./(AllDataRCC(:,(2*ncomp+4)).^2); %get weights for aS: weights = (1/(sigma(aS)^2)
wtaSxaS = wtaS.*aSs; %calc weights x aS
AvgDataRCC(:,2) = (nansum(wtaSxaS,1)./nansum(wtaS,1)); %calc weighted averages for aS
AvgDataRCC(:,(2*ncomp+2)) = sqrt(1./(nansum(wtaS,1))); %calc sigmas

%now tauL
tauLs = AllDataRCC(:,(ncomp+1)); %pick out tauL values
wttauL = 1./(AllDataRCC(:,(2*ncomp+5)).^2); % tauL: weights = (1/(sigma(tauL)^2)
wttauLxtauL = wttauL.*tauLs; %calc weights x tauL
AvgDataRCC(:,(ncomp+1)) = (nansum(wttauLxtauL,1)./nansum(wttauL,1)); %calc weighted
%averages for tauL
AvgDataRCC(:,(2*ncomp+3)) = sqrt(1./(nansum(wttauL,1))); %calc sigmas

%now tauS
tauSs = AllDataRCC(:,(ncomp+2)); %pick out tauS values
wttauS = 1./(AllDataRCC(:,(2*ncomp+6)).^2); %tauS: weights = (1/(sigma(tauS)^2)
wttauSxtauS = wttauS.*tauSs; %calc weights x tauS

```

```

AvgDataRCC(:,(ncomp+2)) = (nansum(wttauSxtauS,1)./nansum(wttauS,1)); %calc weighted
%averages for tauS
AvgDataRCC(:,(2*ncomp+4)) = sqrt(1./(nansum(wttauS,1))); %calc the new sigmas (to report
%with weighted averages)

%lastly, tauAvg - slightly different from the others above: calculate tauAvg from the weighted
%average amps and taus:
amps = AvgDataRCC(:,1:ncomp); %isolate amps
taus = AvgDataRCC(:,(ncomp+1):(2*ncomp)); %isolate taus
AvgDataRCC(:,(4*ncomp+1)) = amps(:,1).*taus(:,1) + amps(:,2).*taus(:,2); %calc tau avg
%Calculating sigma requires derivatives like it did for aL and aS but the denominator can be
%ignored since it should add up to 1 in the eqn of amp wted tauAvg (fractional amps summed)
AvgDataRCC(:,(4*ncomp+2))=(AvgDataRCC(:,3).^2).*(AvgDataRCC(:,5).^2)+(AvgDataRCC(
(:,1).^2).*(AvgDataRCC(:,7).^2)+(AvgDataRCC(:,4).^2).*(AvgDataRCC(:,6).^2)+(AvgDataRC
C(:,2).^2).*(AvgDataRCC(:,8).^2);
AvgDataRCC(:,(4*ncomp+2)) = sqrt(AvgDataRCC(:,(4*ncomp+2))); %calc sigmas

%create folders for bootstrapping plots to be stored in
cd(parent);
mkdir('Bootstrapping Plots');
plots = [parent,'\Bootstrapping Plots'];
cd(plots);
mkdir('aLplots');
aLplots = [plots,'\aLplots'];
mkdir('aSplots');
aSplots = [plots,'\aSplots'];
mkdir('tauLplots');
tauLplots = [plots,'\tauLplots'];
mkdir('tauSplots');
tauSplots = [plots,'\tauSplots'];
mkdir('tauAvgplots');
tauAvgplots = [plots,'\tauAvgplots'];
cd(parent);

%bootstrap each lifetime component, amplitude and tau avg for each snapshot
%=====
%amps are dependent on ea/o so can't bootstrap them separately. Instead, find the error on aS
%since it will be larger, then use that to back out errors on aL

%first, aS/(aL + aS) and then calc aL/(aL + aS) and the relevant parameters from the aS results

data = zeros(nleaves,1);

%=====
%Bootstrapping of measurements over 20 samples (steps RCC already chosen)
%=====

```

```

clear snap;
clear step;
AllDataCIs = zeros((2*ncomp+1),5,nsnaps);
%5 params(amps,taus,tau avgs), 5 things (bCI avg, bCI low, bCI high, and h and p from
%lilliefores test)stored for each param, n snaps

%first aS and aL:
for snap = 1:nsnaps
    %Now I don't need to normalize bc WtAllDataRCC was from AllDataNew (fractional amps)
    for sam = 1:nleaves
        data(sam,1) = AllDataRCC(sam,snap,ncomp); %data is fractional aS values for n samples
    end
    %deal with possible NaNs in data
    NaNs = isnan(data); %new vector of 0s where there aren't NaNs and 1s where there are NaNs
    NaNi = find(NNaNs ==1); %get indices of NaNs
    for ind = 1:size(NNaNi)
        if NaNi(ind)==1
            data(NNaNi(ind)) = ((data(NNaNi(ind)+2)) + (data(NNaNi(ind)+1)))/2;
        elseif NaNi(ind)==nleaves
            data(NNaNi(ind)) = ((data(NNaNi(ind)-1)) + (data(NNaNi(ind)-2)))/2;
        else
            data(NNaNi(ind)) = ((data(NNaNi(ind)-1)) + (data(NNaNi(ind)+1)))/2;
        end
    end
end

nSamples = 100000; %number of sets of nleaves aL values to generate from the nleaves
%measured values

% Lilliefors test - see whether my data had a near Gaussian distribution: h == 0 means Gauss, h
%== 1 means not. Very low p-val indicates unreliable h
[h,p] = lillietest(data);
lillie = [h,p];
%calc CIs from bootstrapping and store them in an array
cd(code);
[bCI, samples] = bootstrap(data, nSamples); %bootstrap to get bCIs

cd(parent);
AllDataCIs(ncomp,1:3,snap) = bCI; %store bCI values
AllDataCIs(ncomp,4:5,snap) = lillie; %store h and p from lilliefores test

% Plot Input Data vs prob of measuring the measured value, as determined from CI from
%bootstrapping
clear h;
h = figure; % figure comes with preset axes
ax = axes; % axes is a command to give the axis from figure
hist(ax,data);

```

```

line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r'); %low
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r'); %high
title('measured tauS amplitude vs probability of getting measured value calculated from
bootstrapping CIs');
xlabel('measured tauS amplitude normalized as a fraction of total amplitude');
ylabel('Probability of measuring aS');
filename = ['WtaSmeasVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(aSplots); %enter plots folder
saveas(h,filename);
close(h); %closes the figure. Plot the average aS values calculated from 100,000 different
%'samples' generated by bootstrapping measured aS values vs the prob of finding that aS value
%as determined from the bootstrapped CIs
clear h;
cd(parent);
h = figure;
ax = axes;
hist(ax,nanmean(samples, 2)); %plots the avg value of each of the 20 samples from the
%100,000 sets of 20 samples generated by bootstrapping on axes set above
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('Distribution of Means from Bootstrap Sampling vs. probability from bootstrapping CIs');
xlabel('Distribution of means of normalized fractional aS from bootstrapping');
ylabel('Pr(Mean)');
filename = ['WtaSmeansFrmBsVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(aSplots);
saveas(h,filename);
close (h);
%Get the values for aL from these aS values and repeat
%the other bits of the process as necessary
cd(parent);
AllDataCIs(1,1,snap) = 1-bCI(1); %avg aL = 1 - avg aS
AllDataCIs(1,2,snap) = 1-bCI(3); %aL lb = 1 - aS ub
AllDataCIs(1,3,snap) = 1-bCI(2); %aL ub = 1 - aS lb
AllDataCIs(1,4:5,snap) = lillie;
% Plot Input Data vs prob of measuring the measured value as determined from CI
clear h;
h = figure; % figure comes with preset axes
ax = axes; % axes is a command to give the axis from figure
hist(ax,(1-data));
line([(1-bCI(3)) (1-bCI(3))],get(ax,'YLim'), 'Color', 'r');
line([(1-bCI(2)) (1-bCI(2))],get(ax,'YLim'), 'Color', 'r');
title('measured tauL amplitude vs probability of measuring tauL value calculated from
bootstrapping CIs');
xlabel('tauL amp, calculated from measured tauS amp, normalized as a fraction of total
amplitude');
ylabel('Probability of measuring aL');

```

```

filename = ['WtaLmeasVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(aLplots);
saveas(h,filename);
close(h);
% Plot avg aL values calculated by subtracting 100,000 different 'samples' generated by
%bootstrapping measured aS values from 1, vs the prob of finding that aL value as determined
%from the bootstrapped CIs (calculated from bootstrapped CIs of aS).
cd(parent);
clear h;
h = figure;
ax = axes;
hist(ax,(1-nanmean(samples, 2))); %plots the avg value of each of the 20 samples from the
%100,000 sets of 20 samples generated by bootstrapping on axes set above
line([(1-bCI(3)) (1-bCI(3))],get(ax,'YLim'), 'Color', 'r');
line([(1-bCI(2)) (1-bCI(2))],get(ax,'YLim'), 'Color', 'r');
title('Distribution of aL Means calc from Bootstrap Sampling of vs. probability from
bootstrapping CIs');
xlabel('Distribution of means of normalized fractional aL calculated from bootstrapping aS');
ylabel('Pr(Mean)');
filename = ['WtaLmeansFrmBsVprFrmBCIsSnapshot',int2str(snap),'.fig'];
cd(aLplots);
saveas(h,filename);
close(h);
cd(parent);
end

%now tauL:
clear snap;
clear sam;
clear ind;
clear NaNs;
clear NaNi;

for snap = 1:nsnaps
    for sam = 1:nleaves
        data(sam,1) = AllDataRCC(sam,snap,(ncomp+1)); %data is tauL values for the n samples
    end
%need to deal with possible NaNs in data
NaNs = isnan(data); %new vector: 0s where there aren't NaNs and 1s where there are NaNs
NaNi = find(NaNi ==1); %get indices of NaNs
for ind = 1:size(NaNi)
    if NaNi(ind)==1
        data(NaNi(ind)) = ((data(NaNi(ind)+2)) + (data(NaNi(ind)+1)))/2;
    elseif NaNi(ind)==nleaves
        data(NaNi(ind)) = ((data(NaNi(ind)-1)) + (data(NaNi(ind)-2)))/2;
    else

```

```

        data(NaNi(ind)) = ((data(NaNi(ind))-1)) + (data(NaNi(ind)+1))/2;
    end
end

nSamples = 100000; %the number of sets of nleaves aL values to generate from the nleaves
%measured values

% Lilliefors test - see whether my data had a near Gaussian distribution - h == 0 means Gauss, h
== 1 means not. Very low p-val indicates unreliable h
[h,p] = lillietest(data);
lillie = [h,p];
%calc CIs from bootstrapping and store them in an array
cd(code);
[bCI, samples] = bootstrap(data, nSamples); %bootstrap to get bCIs

cd(parent);
AllDataCIs((ncomp+1),1:3,snap) = bCI; %store bCI high and low
AllDataCIs((ncomp+1),4:5,snap) = lillie; %store h and p

% Plot Input Data vs prob of measuring the measured value as determined from confidence
%interval from bootstrapping
clear h;
h = figure; % figure comes with preset axes
ax = axes; % axes is a command to give the axis from figure
hist(ax,data);
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('measured tauL vs probability of measuring tauL calculated from bootstrapping CIs');
xlabel('measured tauL');
ylabel('Probability of measuring tauL');
filename = ['WttauLmeasVprFrm bCIsSnapshot', int2str(snap), '.fig'];
cd(tauLplots); %enter plots folder
saveas(h,filename);
close(h); %closes the figure. Plot the average aS values calculated from 100,000 different
%'samples' generated by bootstrapping measured aS values vs the prob of finding that aS value
%as determined from the bootstrapped CIs
clear h;
cd(parent);
h = figure;
ax = axes;
hist(ax,nanmean(samples, 2)); %plots the avg value of each of the 20 samples from the
%100,000 sets of 20 samples generated by bootstrapping on axes set above
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('Distribution of Means from Bootstrap Sampling vs. probability from bootstrapping CIs');
xlabel('Distribution of means of tauL from bootstrapping');

```

```

ylabel('Pr(Mean tauL)');
filename = ['WttauLmeansFrmBsVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(tauLplots);
saveas(h,filename);
close (h);
cd(parent)
end

%now for tauS:
clear snap;
clear sam;
clear ind;
clear NaNs;
clear NaNi;

for snap = 1:nsnaps
    for sam = 1:nleaves
        data(sam,1) = AllDataRCC(sam,snap,(ncomp+2));
    end
    %need to deal with possible NaNs in data
    NaNs = isnan(data); %new vector: 0s where there are no NaNs and 1s where there are NaNs
    NaNi = find(NNaNs ==1); %get indices of NaNs
    for ind = 1:size(NNaNi) %if NaNs in the data, replace with the average of two nearby values
        if NaNi(ind)==1
            data(NNaNi(ind)) = ((data(NNaNi(ind)+2)) + (data(NNaNi(ind)+1)))/2;
        elseif NaNi(ind)==nleaves
            data(NNaNi(ind)) = ((data(NNaNi(ind)-1)) + (data(NNaNi(ind)-2)))/2;
        else
            data(NNaNi(ind)) = ((data(NNaNi(ind)-1)) + (data(NNaNi(ind)+1)))/2;
        end
    end
end

nSamples = 100000; %the number of sets of 20 aL values to generate from the 20 measured
%values Lilliefors test - see whether my data had a near Gaussian distribution - h == 0 means
%Gauss, h == 1 means not. Very low p-val indicates unreliable h
[h,p] = lillietest(data);
lillie = [h,p];
%calc CIs from bootstrapping and store them in an array
cd(code);
[bCI, samples] = bootstrap(data, nSamples); %bootstrap to get bCIs

cd(parent);
AllDataCIs((ncomp+2),1:3,snap) = bCI; %store bCIs high and low
AllDataCIs((ncomp+2),4:5,snap) = lillie; %store h and p

```

```

% Plot Input Data vs prob of measuring the measured value as determined from confidence
%interval from bootstrapping
clear h;
h = figure; % figure comes with preset axes
ax = axes; % axes is just a command to give the axis from figure
hist(ax,data);
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('measured tauS vs probability of measuring tauS calculated from bootstrapping CIs');
xlabel('measured tauS');
ylabel('Probability of measuring tauS');
filename = ['WttauSmeasVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(tauSplots); %enter plots folder
saveas(h,filename);
close(h); %closes the figure so I don't have a million figures at the end... Plot the average aS
%values calculated from 100,000 different 'samples' generated by bootstrapping measured aS
%values vs the prob of finding that aS value as determined from the bootstrapped CIs
clear h;
cd(parent);
h = figure;
ax = axes;
hist(ax,nanmean(samples, 2)); %plots the avg value of each of the 20 samples from the
%100,000 sets of 20 samples generated by bootstrapping on axes set above
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('Distribution of Means from Bootstrap Sampling vs. probability from bootstrapping CIs');
xlabel('Distribution of means of tauS from bootstrapping');
ylabel('Pr(Mean tauS)');
filename = ['WttauSmeansFrmBsVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(tauSplots);
saveas(h,filename);
close (h);
cd(parent)
end

% and lastly, tauAvg:
clear snap;
clear sam;
clear ind;
clear NaNs;
clear NaNi;

for snap = 1:nsnaps
    for sam = 1:nleaves
        data(sam,1) = AllDataRCC(sam,snap,(4*ncomp+5));
    end
end

```

```

%need to deal with possible NaNs in data
NaNs = isnan(data); %new vector: 0s where there are no NaNs and 1s where there are NaNs
NaNi = find(NaNi ==1); %get indices of NaNs
for ind = 1:size(NaNi) %if NaNs in the data, replace with the average of two nearby values
    if NaNi(ind)==1
        data(NaNi(ind)) = ((data(NaNi(ind)+2)) + (data(NaNi(ind)+1)))/2;
    elseif NaNi(ind)==nleaves
        data(NaNi(ind)) = ((data(NaNi(ind)-1)) + (data(NaNi(ind)-2)))/2;
    else
        data(NaNi(ind)) = ((data(NaNi(ind)-1)) + (data(NaNi(ind)+1)))/2;
    end
end
end

nSamples = 100000; %the number of sets of nleaves aL values to generate from the nleaves
%measured values Lilliefors test - see whether my data had a near Gaussian distribution - h == 0
%means Gauss, h == 1 means not. Very low p-val indicates unreliable h
[h,p] = lillietest(data);
lillie = [h,p];
%calc CIs from bootstrapping and store them in an array
cd(code);
[bCI, samples] = bootstrap(data, nSamples); %bootstrap to get bCIs

cd(parent);
AllDataCIs(5,1:3,snap) = bCI; %store bCIs high and low
AllDataCIs(5,4:5,snap) = lillie; %store h and p

% Plot the Input Data vs probability of measuring the measured value as determined from
%confidence interval from bootstrapping
clear h;
h = figure; % figure comes with preset axes
ax = axes; % axes is just a command to give the axis from figure
hist(ax,data);
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('tauAvg calc from fit params of TCSPC data vs probability of tauAvg calculated from
bootstrapping CIs');
xlabel('measured tauAvg');
ylabel('Probability of measuring tauAvg');
filename = ['WttauAvgmeasVprFmbCIsSnapshot', int2str(snap), '.fig'];
cd(tauAvgplots); %enter plots folder
saveas(h,filename);
close(h); %closes the figure so I don't have a million figures at the end... Plot the average aS
%values calculated from 100,000 different 'samples' generated by bootstrapping measured aS
%values vs the prob of finding that aS value as determined from the bootstrapped CIs
clear h;
cd(parent);

```

```

h = figure;
ax = axes;
hist(ax,nanmean(samples, 2)); %plots the avg value of each of the 20 samples from the
%100,000 sets of 20 samples generated by bootstrapping on axes set above
line([bCI(2) bCI(2)],get(ax,'YLim'), 'Color', 'r');
line([bCI(3) bCI(3)],get(ax,'YLim'), 'Color', 'r');
title('Distribution of Means from Bootstrap Sampling vs. probability from bootstrapping CIs');
xlabel('Distribution of means of tauAvg from bootstrapping');
ylabel('Pr(Mean tauAvg)');
filename = ['WttauAvgmeansFrmBsVprFrmBCIsSnapshot', int2str(snap), '.fig'];
cd(tauAvgplots);
saveas(h,filename);
close (h);
cd(parent)
end
end

```

getstepsRCCv2.m

%getstepsRCCv2 is a function that will pull out data for the step with the longest average
%lifetime for each snapshot
%Author: Michelle Leuenberger
%Version: 2
%May 4th, 2016

%Description: This function will take AllData array from the FitFramework script and pick out
%the steps with the reaction centers closed (the steps with the longest average lifetime)

%NOTE: this only works if you labeled folders appropriately! I.e. wt01, wt02, etc. when you ran
%FitFramework. Otherwise, you will need the version of stepsRCC with a legend in it (works
%for 20 samples)

%Inputs:

%AllData = the array generated by FitFramework
%nsnaps = number of snapshots per TCSPC run
%sample = leaf you want to look at
%nsteps = number of steps per snapshot

%Outputs:

%stepsRCC = an array that contains the step found to have the longest avg lifetime for each
%snapshot - i.e. the step with the rxn ctrs closed

function [stepsRCC] = getstepsRCCv2(AllData,nsnaps,sample,nsteps,ncomp)

stepsRCC = zeros(1,nsnaps); %array to put steps RCC in
clear **snap**;
clear **step**;

for snap = 1:nsnaps
 tauAvgmax = 0.2;
 for step = 1:nsteps
 if AllData(sample,snap,step,(ncomp*4+5)) > tauAvgmax
 tauAvgmax = AllData(sample,snap,step,(ncomp*4+5));
 stepsRCC(:,snap) = step;
 end
 end
 clear **step**;
end
stepsRCC(stepsRCC==0)=1; % gets rid of zeros in the stepsRCC array due to blank snapshots
end