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Development of Time-Resolved Spectroscopic Tools to Study the Dynamics of Photoprotective Quenching in Plants

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Publication Date
2014

Peer reviewed|Thesis/dissertation
Development of Time-Resolved Spectroscopic Tools to Study the Dynamics of Photoprotective Quenching in Plants

by

Emily Jane Sylak-Glassman

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 Krishna K. Niyogi
Professor Carlos Bustamante

Spring 2014
Development of Time-Resolved Spectroscopic Tools to Study the Dynamics of Photoprotective Quenching in Plants

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by

Emily Jane Sylak-Glassman
Abstract

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Plants experience rapid fluctuations in light intensity due to intermittent shading. While under low light conditions, efficient absorption of light is important, under high light conditions, this efficient light harvesting can result in excess energy that leads to photodamage. To prevent this photodamage, plants have a set of mechanisms called non-photochemical quenching (NPQ) that allow the plant to dissipate the excess energy harmlessly as heat.

While NPQ has been studied for decades, there is still much we do not know about how NPQ is triggered, the changes in the photosynthetic apparatus that occur during NPQ, and the photophysical mechanisms that quench excess energy. NPQ is difficult to study both because of the large number of feedback loops and processes that contribute to NPQ and because the photosynthetic apparatus changes as NPQ turns on and off. This thesis describes the development of new time-resolved spectroscopic tools to help elucidate the proteins and processes important in NPQ.

One of the techniques developed is a tool to measure the chlorophyll fluorescence lifetimes of whole leaves as they acclimate to changing light conditions. Chlorophyll is the main pigment in photosynthetic organisms, and its excited state relaxation dynamics reveal information about the energy transfer network in plants. The chlorophyll fluorescence lifetime gives additional information about quenching than the most commonly used probe of NPQ, the chlorophyll fluorescence yield. This tool is able to examine both the nanosecond-timescale and seconds- to minutes-timescale of quenching in vivo.

This thesis also describes the application of the fluorescence lifetime technique to study different types of NPQ. Energy-dependent quenching (qE) is the largest and fastest component of NPQ. By comparing the shape of the fluorescence decay curves for plants with and without PsbS, a key protein in qE, it is possible to demonstrate that the presence of the protein PsbS changes only the amount of qE and the speed of qE turn-on and turn-off, not the type of quenching. This suggests that PsbS catalyzes qE. In contrast, the presence of the enzyme violaxanthin de-epoxidase and the carotenoid zeaxanthin do seem to affect the type of quenching. The fluorescence lifetime technique is also applied to study a new family of mutants that lack the protein SOQ1. Here, the fluorescence lifetime measurements confirm that the lack of SOQ1 confers extra quenching as opposed to extra photobleaching. Furthermore, the technique is able to show that SOQ1 reduces the timescale of long-term quenching.
that normally turns on in approximately 45 minutes by a factor of two. Additionally, a new mutant in the *soql* family shows the highest quenching of any mutant previously studied, and may be a model organism to study energy transfer when Photosystem II reaction centers are open.

The development of another tool, a super-resolution stimulated emission depletion (STED) fluorescence lifetime imaging (FLIM) microscope, is also discussed. The goal of this tool was to be able to image the thylakoid membrane of plants using chlorophyll fluorescence. Chlorophyll from different protein environments would be distinguished by their fluorescence lifetime, and the STED technique would provide the resolution needed to distinguish the different parts of the thylakoid membrane. However, different types of STED-based imaging were unable to achieve super-resolution with chlorophyll.

Time-resolved measurements of chlorophyll fluorescence offer a way to sensitively probe the dynamics of quenching in plants. In the future, the fluorescence lifetime technique can be applied to other processes and components of NPQ. In addition, the lessons learned in the development of both the fluorescence lifetime technique and the microscopy technique can be used to inform the development of other tools.
For John, who has been with me every step of the way.
Acknowledgements

I would like to thank my advisor, Professor Graham R. Fleming, for his support. Graham’s confidence in me inspired me to trust my instincts and take on much more responsibility than I would have ever believed I could. The freedom to make my own decisions allowed me to become an independent scientist, and I am extremely grateful.

I would also like to thank Professor Laurie Butler. Laurie’s mentorship has extended beyond my undergraduate education. Her continued support and belief in me is overwhelming.

I have been incredibly fortunate to learn from many other Fleming group graduate students, including Tessa Calhoun, Jacquelyn Burchfield, Doran Bennett, Kapil Amarnath, Julia Zaks, and Eleonora De Re. I would also like to thank Jahan Dawlaty for his mentorship during my first years of graduate school. I have never met anyone who could explain everything from non-linear spectroscopy to Fourier optics with such clarity and patience.

I have also been very lucky to work with wonderful collaborators. I would like to thank Professor Kris Niyogi, Matthew Brooks, and Alizée Malnoë. Working with Alizée has been a true privilege— I have come away from every conversation inspired to learn more and experiment more, and feel lucky to have gained a wonderful colleague and friend. Professor Stefan Hell and the diploma and graduate students, post-doctoral researchers, and staff of the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry were unfailingly welcoming and helpful during my time in their labs.

I am grateful for the financial support from the National Science Foundation Graduate Research Fellowship Program and the Department of Energy.

I would also like to thank my family, all of whom have been incredibly supportive throughout graduate school. To my dad, who used his amazing logic skills to talk me through research problems completely outside his field and from 3000 miles away; to my mom, who inspired me to tough it out during the slow periods and trust my instincts; to my sister, Julia, who is my best friend and makes me laugh harder than anyone, I cannot thank the three of you enough. I would also like to thank my grandparents, who believed in me fiercely and showed an overwhelming amount of love and support. I cannot express enough gratitude to my friends, especially Matthew Chapman, Doran and Nina Bennett, Eleonora De Re, Melinda Fricke, Maggie Yandell, and Annelise Beck, who, over the past five years, have become family.

Most of all, thank you to my husband, John, who has been my biggest supporter. John’s kindness, insight, humor, smarts, patience, understanding, joie de vivre, and Mexican cooking have made California a home and filled my time in graduate school with wonderful memories.
3.4.1 Average fluorescence lifetimes of wild-type and qE-mutants .......................... 38
3.4.2 Differences in the average fluorescence lifetimes between pairs of plants ........................................ 40
3.4.3 Fluorescence decay comparisons ..................................................... 42
3.5 Discussion ................................................................................. 46
3.6 Conclusions ............................................................................. 49
3.7 Supplemental ........................................................................... 49

4 Characterizing a new type of NPQ mutant ........................................................................ 52
4.1 Collaborators ............................................................................ 52
4.2 Introduction ................................................................................ 52
4.2.1 Thioredoxins ........................................................................... 53
4.2.2 Previous experiments on soq1 mutants .............................................. 53
4.2.3 Fluorescence lifetime measurements on soq1 mutants ...................... 54
4.3 Methods ...................................................................................... 54
4.4 Results ......................................................................................... 55
4.4.1 Investigation of quenching in plants with and without SOQ1 .............. 55
4.4.2 Investigating whether high light can induce enhanced quenching ........ 59
4.4.3 Average fluorescence lifetime of A164 ............................................ 62
4.5 Discussion .................................................................................... 69
4.6 Conclusions ................................................................................ 70
4.7 Supplemental .............................................................................. 71
4.7.1 Investigating the D101 mutant ....................................................... 71
4.7.2 Fits to the induction of NPQ in soq1 npq4 and npq4 ......................... 72

5 Building a STED-FLIM microscope .................................................................................. 74
5.1 Collaborators .............................................................................. 74
5.2 Introduction ................................................................................ 74
5.3 Theory of STED Microscopy ............................................................ 75
5.4 Design ......................................................................................... 83
5.5 Results ......................................................................................... 89
5.5.1 Pulsed STED Imaging ................................................................. 89
5.5.2 CW STED Imaging ................................................................. 91
5.5.3 Time-gated STED Imaging ...................................................... 91
5.6 Discussion .................................................................................... 93
5.7 Conclusions ................................................................................ 95

6 Conclusions .................................................................................. 96
Chapter 1

Introduction


1.1 Light harvesting in plants

Photosynthesis, the collection of light and conversion to chemical energy, takes place in the thylakoid membrane which is held inside the chloroplast [1]. There are two different configurations of the thylakoid membrane: folded and enclosed membranes that are stacked, called the grana, and unfolded regions called the stroma lamellae (for review, see [2]). This is shown in Fig. 1.1.

The thylakoid membrane is the most densely packed membrane in nature, filled with approximately 70% protein, and 80% in the grana stacks [4]. There are two photosystems, Photosystem I and Photosystem II. Photosystem I is found mostly in the stroma lamellae, and Photosystem II is found mostly in the grana. A schematic of the grana, as viewed from the top can is shown in Fig. 1.2. The most abundant protein in the thylakoid is Light Harvesting Complex II (LHCII) [5]. Sunlight is absorbed principally by chlorophyll bound in LHCII and transferred to Photosystem II (PSII), where a charge separation occurs, converting the excitation energy to chemical energy.

When light is transferred to PSII, it excites a strongly coupled pair of chlorophyll molecules called P680 due to its absorption peak at 680 nm. Upon excitation, P680 donates an electron to pheophytin (Pheo). P680 is reduced with an electron generated from water splitting and transferred via a tyrosine residue, YZ. The pheophytin transfers the electron to the primary quinone, QA, which then transfers the electron to the secondary quinone, QB. Unlike QA, which is a single electron acceptor, QB accepts two electrons from two cycles of charge separation. Once it accumulates two electrons, it becomes reduced with two protons from outside the stromal side of the membrane, and unbinds. An oxidized plastoquinone takes its place. For a review of this process, see [6]. This is shown in Fig. 1.3. The reduced plastoquinone diffuses in the membrane, transferring electrons downstream, eventually reducing NADP+ to NADPH.
1.1. Light harvesting in plants

When the plastoquinone pool is overly reduced, an oxidized plastoquinone is not available to bind to PSII. This results in the electron remaining on $Q_A$ (for review, see [7]). Electrostatic repulsion from $Q_A^-$ is thought to repel Pheo$^-$ [8]. The reaction centers are said to be closed at this point because they cannot accept more excitation [9].

The closing of reaction centers is dangerous for both PSII and other photosynthetic pigment protein complexes (PPCs) due to the formation of damaging reactive oxygen species. When PSII reaction centers are open, energy transfer to the reaction center is the dominant pathway, with excitation diffusion within a PSII supercomplex occurring within approximately 110 ps [10]. Excited chlorophyll in PPCs can also fluoresce, which occurs on a timescale of nanoseconds. Additionally, it can undergo intersystem crossing to a triplet state. This also occurs on timescale of nanoseconds. Triplet chlorophyll can react with ground state triplet oxygen to form singlet oxygen. The relaxation pathways for chlorophyll are shown in Fig. 1.4. Singlet oxygen is extremely reactive and can cause significant damage (for review, see [11]). In addition to intersystem crossing, triplet states can be formed through charge recombination. In the case of PSII, triplet states form via charge recombination between Pheo$^-$ and P680$^+$ [12]. To prevent this damage from occurring, plants have additional chlorophyll relaxation pathways that can be turned on in response to excess light. These quenching mechanisms are called non-photochemical quenching (NPQ).
Figure 1.2: A top view schematic of the grana membrane is shown below. Photosystem II (PSII) complexes are shown in blue and Light Harvesting II (LHCII) complexes are shown in green.
1.1. Light harvesting in plants

Introduction

Figure 1.3: The electron transfer in PSII is designated by the arrows.
Figure 1.4: Excitation of chlorophyll occurs from $S_0$ to $S_1$. Once excited, chlorophyll can relax via energy transfer to the $S_1$ state of neighboring chlorophylls, it can fluoresce, or it can undergo intersystem crossing to the triplet state $T_1$. The lifetime of $T_1$ is long because relaxation to $S_0$ is spin-forbidden. However, it can relax via conversion of $^3O_2$ to $^1O_2$. 

Intrinsic decay pathways

Energy transfer $S_1$

S$_1$ of neighboring chlorophyll

intersystem crossing

$T_1$

flurescence

$^3O_2$

$^1O_2$
1.2 Non-photochemical quenching (NPQ)

By dissipating energy when PSII reaction centers are closed, NPQ allows plants to respond to changes in light intensity. The ability of plants to respond appropriately to light intensity has implications for plant fitness. Plants that are unable to turn NPQ on quickly enough or strongly enough are unable to produce as many fruits or seeds as wild-type plants [13]. In addition, plants that are unable to turn NPQ off quickly enough waste energy that could be used for productive photosynthesis [14].

The mechanisms of NPQ in plants are generally broken down into energy-dependent quenching (qE), state transitions (qT), inhibition quenching (qI) [15], and zeaxanthin-dependent quenching (qZ) [16]. The classification of different contributors to quenching is somewhat controversial. Mechanisms are sometimes grouped by the timescales of activation and relaxation [17]. The response times of qE are seconds to minutes, of qZ are 15-30 minutes, of qT are tens of minutes [18], and of qI are hours. Alternatively, the different types of quenching are sometimes grouped by their molecular trigger(s). qE is defined to be a pH-dependent response [19], qZ to be a slowly relaxing zeaxanthin-dependent quenching [16], qT by phosphorylation of LHCII [20], and qI by PSII damage [15].

NPQ is interesting for a number of reasons. In qE, the speed with which quenching can turn on and off is faster than can be accounted for by changing gene expression, which can only take place within tens of minutes [21]. From an engineering standpoint, the ability of a plant to quickly dynamically regulate the behavior of the membrane without modifying its protein composition is particularly impressive. The design principles of this regulation would be useful as a blueprint for artificial photosynthetic systems. Additionally, in large bioreactors or ponds of single-celled photosynthetic organism cultures and in multi-layered plants, the amount and color of the light that the chloroplasts experience near the light source differs significantly from the light amount and color experienced far from the source [22, 23]. qT allows plants to respond to light of different wavelengths [24], which suggests that adapting to different light spectra may help in engineering crops or cultures to optimize biomass or production of a natural product.

While NPQ has been studied for over 45 years, we do not yet have a complete understanding of the signaling pathways responsible for triggering the different components of NPQ. We also have a relatively poor understanding of what changes take place in the thylakoid that allow the plant to switch between a quenching state and a non-quenching state. Lastly, we do not know exactly what photophysical mechanisms are responsible for dissipating the energy from chlorophyll, and where that quenching occurs. Quenching through chlorophyll-chlorophyll interactions [25, 26] and chlorophyll-carotenoid interactions [27, 28, 29] have both been proposed.
1.3 Techniques and tools to study NPQ

1.3.1 NPQ Mutants

Plant mutants that display enhanced or inhibited quenching have aided in identifying the components that are necessary to see a full NPQ response. Many of these mutants were created by randomly mutating *Arabidopsis thaliana* seeds by fast neutron bombardment, treatment with ethyl methanesulfonate (EMS), or transfer DNA. Seedlings are selected and characterized by their fluorescence yield, often with a video imaging technique used by Niyogi and co-workers that allows for rapid visualization of NPQ on a large number of mutagenized seedlings [31]. Plants with a low or high amount of NPQ compared to wild-type can then be further characterized. A partial list of NPQ mutants is in Tab. 1.1.

Because of the complexity and interconnectedness of the thylakoid membrane, removing one component, such as a pigment or a protein, may cause other components in the membrane to compensate in a manner that is unpredictable and challenging to characterize. One example of this is the mutant *npq1*, which cannot convert violaxanthin to zeaxanthin [31] and shows reduced quenching. However, the mutation does not block the biosynthesis of zeaxanthin from β-carotene. Therefore, while *npq1* has a strongly reduced amount of zeaxanthin, some zeaxanthin and antheraxanthin are still present. In the case of *npq2*, which lacks zeaxanthin epoxidase, zeaxanthin accumulates even in the dark, so quenching components related to qZ are always present in the *npq2* mutant.

Another caution in using mutants is that changing one gene may have unintended consequences on the greater photosynthetic apparatus. For instance, knocking out PsbS as in *npq4* could change the properties of the thylakoid membrane that affect more processes than just qE. PsbS has been shown to affect the stacking of the grana membranes [32], and to affect the distance between PSII centers upon illumination [33]. These changes have not been shown to be directly related to qE, but they complicate the interpretation of the role of PsbS. As another example, the altered qE dynamics of the *lut2* mutant, which lacks lutein, may be due to the misfolding of light harvesting proteins rather than to a change in the qE mechanism [34]. Nonetheless, NPQ mutants have provided a powerful tool for studying the components and mechanism of NPQ.
<table>
<thead>
<tr>
<th>NPQ component</th>
<th>name</th>
<th>mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>qE</td>
<td>npq4 [35]</td>
<td>lacks PsbS function, low qE</td>
</tr>
<tr>
<td></td>
<td>npq4-E122Q [36]</td>
<td>one of two lumen-exposed glutamate residues mutated to glutamine, moderate qE</td>
</tr>
<tr>
<td></td>
<td>npq4-E226Q [36]</td>
<td>one of two lumen-exposed glutamate residues mutated to glutamine, moderate qE</td>
</tr>
<tr>
<td></td>
<td>L5 [37]</td>
<td>over-expresses PsbS, high qE</td>
</tr>
<tr>
<td></td>
<td>L17 [37]</td>
<td>over-expresses PsbS, high qE</td>
</tr>
<tr>
<td>qE and qZ</td>
<td>npq1 [31]</td>
<td>no violaxanthin deepoxidase activity, low quenching</td>
</tr>
<tr>
<td></td>
<td>npq2 [31]</td>
<td>no zeaxanthin epoxidase activity</td>
</tr>
<tr>
<td></td>
<td>lut2 [38]</td>
<td>no production of lutein</td>
</tr>
<tr>
<td></td>
<td>npq1lut2 [39]</td>
<td>see above</td>
</tr>
<tr>
<td></td>
<td>npq4npq1lut2 [37]</td>
<td>see above, no qE or qZ</td>
</tr>
<tr>
<td>qT and qI</td>
<td>stn7 [40]</td>
<td>lacks STN7 kinase</td>
</tr>
<tr>
<td></td>
<td>stn8 [40]</td>
<td>lacks STN8 kinase</td>
</tr>
<tr>
<td></td>
<td>stn7 stn8 [40]</td>
<td>lacks STN7 and STN8 kinases, no state transitions</td>
</tr>
<tr>
<td>Unknown</td>
<td>soq1 [41]</td>
<td>lacks SOQ1, enhanced long-term quenching</td>
</tr>
<tr>
<td></td>
<td>soq1 npq4 [41]</td>
<td>lacks SOQ1 and PsbS, enhanced long-term quenching</td>
</tr>
</tbody>
</table>
1.3.2 Imaging and Microscopy

Assessing the role of protein rearrangement and changes in the thylakoid architectures in NPQ requires tools that can probe the spatial arrangement of proteins in the thylakoid membrane. A rearrangement may be required for the conformational changes that switch a pigment into a quencher. Another theory is that rearrangements may occur to disconnect light harvesting complexes from reaction centers during quenching [42]. Protein dynamics in living systems is typically observed by tagging proteins with fluorophores. However, because most of the proteins of interest are integral membrane proteins and the grana membrane is up to 80% protein [4], such tagging is experimentally difficult. Additionally, the tightly stacked grana membrane prevents the introduction of fluorescent proteins within the stacked region, because the proteins are large compared to the space between membranes. Moreover, there are few fluorescent proteins or dyes whose excitation wavelengths do not coincide with those of carotenoids and chlorophylls, the main light-absorbing pigments in photosynthetic organisms. Because the resolution limit of optical microscopy is \( \sim 200 \text{ nm} \) and because of the difficulties in tagging proteins of interest, protein organization in the thylakoid membrane cannot be currently resolved through confocal optical microscopy. However, qT in algae has been observed through fluorescence lifetime imaging of chlorophyll [43]. However, due to the difficulty in tagging membrane proteins, electron microscopy (EM) and atomic force microscopy (AFM), which are more invasive than optical microscopy and can resolve features on a short length scale, are more often used to image the thylakoid membrane [44, 3].

Imaging of *A. thaliana* mutants using EM (for information about this technique, see references [45, 3]) has recently revealed information about the role of PsbS. Another common EM technique is freeze-fracture electron microscopy, in which thylakoids are frozen and then split along the lipid bilayer such that the transmembrane proteins remain on one side of the split membrane (for review, see [2]). Using freeze-fracture electron microscopy, the Ruban group observed clustering of the light harvesting complexes on the timescales of qE induction [46].

One drawback of using EM techniques is the intensive sample preparation that is required. Negative staining requires fixing and dehydrating the grana, and freeze-fracture images are made with metallic replicas made from the frozen samples. In this way, the sample preparation techniques may impact the arrangement of proteins [44]. To work with more intact systems, there has recently been an effort to image thylakoids using AFM. In AFM, isolated thylakoids are placed on a mica surface exposed to air, and probed with a cantilever. An image is created using the height of the sample for contrast [44]. One drawback of this technique is that having the sample exposed to air rather than immersed in liquid may affect membrane properties [47].

The application of new water-based AFM techniques [48] could probe the native rearrangements that take place in the thylakoid. Such imaging techniques should be extremely valuable for assessing the changes in chlorophyll connectivity in the membrane. In addition, thermodynamic models will be useful for understanding the strength and directionality of energetic interactions between proteins required for causing changes in membrane organization [49, 50, 51]. While imaging techniques are able to probe the arrangement of proteins that
takes place during NPQ, it does not directly probe quenching or give information about the photophysical mechanisms of quenching. To learn more about the energy transfer pathways during quenching, it is necessary to use spectroscopic techniques.

### 1.3.3 Spectroscopy

There are two direct spectroscopic signatures of quenching: a reduced fluorescence yield compared to a dark-adapted plant and a reduced fluorescence lifetime compared to dark-adapted plants. NPQ adds additional relaxation pathways for chlorophyll, which compete with fluorescence, reducing the fluorescence yield and lifetime. Due to the commercial availability of Pulse Amplitude Modulated (PAM) fluorometers, the chlorophyll fluorescence yield is the most frequently used quantity for observing NPQ. PAM fluorometers measure changes in the chlorophyll fluorescence yield as plants acclimate to changing light conditions [52, 53, 54]. The benefit of PAM measurements is that they are able to quantify the amount of quenching in real time non-invasively, and therefore can be used to quickly assay the NPQ response with different mutants, light conditions, and chemical treatments. This technique is discussed further in Chapter 2. The chlorophyll fluorescence lifetime contains more information about the type of quenching than the fluorescence yield. This allows different types of quenching that might result in the same fluorescence yield to be distinguished, as shown in Fig. 1.6. The black line in Fig. 1.6 shows a decay shape when NPQ is off. When NPQ turns on, the fluorescence lifetime decreases. However, there are different quenching scenarios that could result in the same fluorescence yield. Two such quenching scenarios are shown by the gray lines. The dotted line represents a scenario in which a subset of chlorophylls are strongly quenched, but the rest of the chlorophylls are only weakly quenched. The dashed line represents a scenario in which all the chlorophylls are moderately quenched. Until now, there has not been a way to measure fluorescence lifetimes of leaves as they acclimate to changing light conditions. The development of a new technique to measure fluorescence lifetimes in leaves is discussed further in Chapter 2, and its application to different types of quenching is explored in Chapter 3 and Chapter 4.

There are also indirect spectroscopic signatures of quenching. One way to infer quenching is to observe the creation of species or pathways that can lead to quenching. Transient absorption (TA) spectroscopy is a particularly powerful tool to detect non-emissive states. In TA spectroscopy, the absorption spectrum of an excited sample is measured at a range of times following excitation. These absorption spectra are measured at the same range of times without the presence of an excitation. Subtracting the absorption spectrum without the excitation from the absorption spectrum with the excitation gives $\Delta A$, which shows the intermediate states or processes that occur due to excitation [55]. This technique has been used to study the involvement of carotenoids in the photophysical mechanism of energy quenching [56, 29, 57]. While TA spectroscopy has been used to compare the species present for dark-adapted samples and for light-adapted samples, there is not yet a technique that is able to create multiple measurements to follow quenching as it turns on. This would help determine which species form incidentally during quenching, and which species perform a critical role in quenching.
Figure 1.6: The black line shows a representative fluorescence lifetime decay for a sample when NPQ is off. The dotted gray line represents a quenched fluorescence decay with a subset of the population strongly quenched. The dashed gray line represents a quenched fluorescence decay with most of the population moderately quenched. This image is adapted from Figure 7 by Zaks et al. [30].

TA spectroscopy uses changes in absorption on an ultrafast timescale to investigate the creation of states that occur immediately after exciting chlorophyll. There are also changes that occur on a longer timescale of seconds to minutes, and some of these can also be monitored through changes in absorption. One such change is a difference in absorption at 535 nm [58]. The origin of this signal is thought to be due to interactions between two carotenoids (violaxanthin or zeaxanthin) that occur only under qE conditions [59]. There is also a shift in the absorption spectrum of pigments, called the electrochromic shift (ECS), that occurs due the presence of an electric field created during quenching (for a review, see [60]). Raman spectroscopy can also be used as an indirect probe of NPQ. A change in the resonance Raman spectrum of the leaf around 953 cm$^{-1}$ after exposure to light has been observed, which is thought to be due to twisting of a neoxanthin carotenoid in LHCII during quenching [61, 62].

1.4 Development of new tools

While there are many techniques that are currently used to study quenching, there has not been a technique that is able to observe changes in chlorophyll fluorescence lifetime, the only direct and specific measure of NPQ, in leaves. The work described in this thesis discusses the development of new spectroscopic tools to study NPQ in plants through measurements in fluorescence lifetimes. Chapter 2 introduces a new technique to measure chlorophyll fluorescence lifetimes on whole leaves to elucidate how the relaxation dynamics of chlorophyll change as NPQ turns on and off. In Chapter 3, this technique is applied to qE mutants, npq4,
npq1, and npq1 npq4. While these mutants have been studied for over a decade, the technique is able to reveal new information on the roles of PsbS and zeaxanthin in quenching. In Chapter 4, we apply the fluorescence lifetime technique to study a new family of mutants that lack the protein SOQ1 to show that this protein suppresses a long-lived quenching state. Chapter 5 discusses an attempt to add spatial resolution to in vivo fluorescence lifetime measurements. The theory and development of a stimulated emission depletion (STED) fluorescence lifetime imaging (FLIM) microscope to study qI is discussed, as well as the application of different STED-based techniques to imaging isolated thylakoid membranes and de-enveloped chloroplasts.
Chapter 2

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements
2.1 Collaborators

Dr. Julia Zaks and Dr. Kapil Amarnath assisted in the early development stages of the technique. Michelle Leuenberger assisted with some of the data collection. Robert Shih assisted in growing plants for experiments. Dr. José García and Alma Popescu performed the chloroplast isolation.

2.2 Introduction

Plants dynamically regulate the photosynthetic apparatus in response to fluctuations in light intensity. Light absorbed by light harvesting complexes in the Photosystem II (PSII) antenna is transferred to PSII reaction centers, where it is converted to chemical energy through charge separation [1]. At low light intensities, plants have approximately 90% efficiency of charge separation [63]. However, under high light intensities, it is possible to exceed the plant’s capacity for charge separation, which results in the accumulation of excited chlorophyll. This is dangerous, because excited chlorophyll can undergo intersystem crossing to a triplet state [64] which can react with oxygen to form reactive oxygen species that damage PSII reaction centers [65, 66]. To prevent damage from occurring, plants turn on non-photochemical quenching (NPQ), a set of mechanisms that allow the excited chlorophyll to relax safely, dissipating excess energy as heat. Mutant plants that are deficient in NPQ are not able to produce as many seeds or fruits as wild-type [13], indicating that the plant’s ability to perform NPQ affects plant fitness. However, the dynamics of NPQ, not just the amount of NPQ, also impact plant growth; if NPQ remains on when the plant switches from high light to low light conditions, the relaxation pathways from NPQ compete with energy transfer to PSII, reducing the amount of productive photosynthesis possible [14]. In order to understand NPQ in plants, and perhaps improve crop yields, is necessary to monitor the changes in the chlorophyll relaxation pathways.

The amount of NPQ is generally measured through changes in the chlorophyll fluorescence yield. One problem with monitoring the dynamics of NPQ in this way is that processes other than NPQ can change the chlorophyll fluorescence yield. For example, photobleaching and chloroplast movement decrease the fluorescence in the field of view and lower the fluorescence yield. This decrease in fluorescence yield can complicate the interpretation of changes in fluorescence yield in terms of NPQ [67]. An additional challenge with using the chlorophyll fluorescence yield is the limited amount of information it gives about the type of quenching. During NPQ, chlorophyll transfers energy to quenching sites. To understand what is happening during NPQ, it is desirable to know the number and location of these quenching sites, as well as the rates of quenching. The shape of the chlorophyll fluorescence lifetime decay is determined by each of these parameters, and can be used to distinguish between different quenching situations, such as a few strong quenching sites versus many weak quenching sites. In contrast, the chlorophyll fluorescence yield is just determined by the total amount of quenching, not the method of quenching (for a review, see [30]). Because the chlorophyll fluorescence lifetime can be used to distinguish between photobleaching and...
quenching, as well as between different quenching scenarios, it is desirable to be able to monitor NPQ through fluorescence lifetime measurements.

Measuring chlorophyll fluorescence lifetimes on intact photosynthetic systems has been challenging for a number of reasons. One difficulty is in balancing the time required to collect enough photons with the need to make measurements quickly enough such that the fluorescence lifetime does not change during the measurement. Amarnath and co-workers [68] solved this issue by developing a system of computer-controlled shutters to measure fluorescence lifetimes of *Chlamydomonas reinhardtii* (*C. reinhardtii*) every 200 ms as NPQ turns on and off. The shutter system allowed for short measurement durations (80 ms) to ensure that the lifetime did not change significantly during the measurement. The automation of the measurements allowed for the measurements to be repeated on enough aliquots of *C. reinhardtii* to accumulate enough photons [68]. Adapting this technique to intact leaves requires addressing a number of issues. One concern is that due to a high concentration of chloroplasts in a leaf, a portion of the fluorescence emitted from chloroplasts can be reabsorbed by other chloroplasts and then re-emitted, causing an artificially long fluorescence lifetime [69]. Additionally, to measure change in fluorescence lifetime due to NPQ, it is necessary to close the PSII reaction centers during the measurements. The high light required to close the reaction centers in leaves can overwhelm the fluorescence signal or overload the sensitive detectors used to measure the fluorescence. Holzwarth and co-workers [70] designed a sample holder that allows for the closing of reaction centers with simultaneous measurement of fluorescence lifetimes, but it does not give information on the dynamics of the induction and relaxation of NPQ.

In this paper, we describe a new method that is capable of measuring fluorescence lifetimes on detached leaves throughout the induction and relaxation of NPQ. We show that the chlorophyll fluorescence lifetimes are not contaminated by fluorescence that has been absorbed and re-emitted. We discuss the apparatus, illumination, and detection parameters that impact the measured fluorescence lifetimes. Like PAM measurements, this technique captures *in vivo* dynamics of NPQ, but it adds the ability to distinguish between photobleaching and quenching, and between different quenching scenarios.

### 2.3 Methods

Measurements were done on medium-sized *Arabidopsis thaliana* (*A. thaliana*) leaves detached from plants before the stage of bolting. Plants were grown in a growth chamber with 100 $\mu$mol photons·m$^{-2}$·s$^{-1}$ light.

*A. thaliana* plants were each dark-adapted for 30 minutes prior to detaching a leaf. To prevent any plant from having their light cycle disturbed, measurements were performed on multiple plants during each day of data collection so that any given plant would not be dark-acclimated for more than 30 minutes in a 1.5 hour period of time. Between dark adaptation times, plants were placed in a 100 $\mu$mol photons·m$^{-2}$·s$^{-1}$ light chamber for re-light acclimation. Each leaf was selected from the middle of the rosette to control for leaf age and size. After leaf selection, the leaf was immediately placed in a home-built holder.
Due to the difficulty in placing an Arabidopsis thaliana (A. thaliana) leaf attached to the whole plant at the appropriate angle to the laser, actinic light, and detector, a leaf holder was designed. To keep the leaf from overheating or dehydrating, the holder keeps most of the leaf open to air, and allows the leaf to take up water. It is shown in Supplemental Fig. 2.7. While a leaf that is able to uptake water during the measurement shows the same fluorescence lifetime as a leaf attached to a plant, a detached leaf without water is unable to perform the same amount of quenching. This is shown in Supplemental Fig.2.8.

Similar to the set-up described by Amarnath et al., the light exposure of the leaf and detector were controlled by shutters programmed in LabVIEW. The set-up is shown in Figure 2.1. A 532 nm diode pumped laser (Coherent G10) is used to pump a Ti:Sapphire oscillator (Coherent Mira 900) set to 840 nm. The resulting light is frequency-doubled to 420 nm with a β-barium borate (BBO) crystal to excite the Soret band of chlorophyll a (chl a). The beam was then split by a beamsplitter. One portion of the light is directed to a photodiode (PD) to become the SYNC pulse for the time-correlated single photon counting (TCSPC) card (Becker-Hickl SPC-630 and SPC-850). The other portion of the laser light was intermittently blocked by a shutter, but was impinged on the leaf at a 70° angle to the adaxial side of the leaf. A diagram of the angle of laser exposure and detection is shown in Fig. 2.1b. The average power of the laser at the sample was 5 mW with a pulse energy of 66 pJ. The laser power of approximately 5 mW corresponds to approximate intensity of 5000 µmol photons·m⁻²·s⁻¹, which is enough to close reaction centers [71] and is similar to the intensity used in PAM fluorometers [72, 73, 54].

In addition the laser light, an actinic light (Schott KL1500) was used to illuminate the sample. The leaf's exposure to the actinic light was also controlled by a shutter. The actinic light intensity was set at either 500 µmol photons·m⁻²·s⁻¹ or 1200 µmol photons·m⁻²·s⁻¹ for experiments. The light was collimated by a lens, forming a 1 cm diameter illumination spot size at a 45° angle to the adaxial side of the leaf.

Detection occurred at a 45° angle to the adaxial side of the leaf and at a 65° angle to the laser. Fluorescence was collimated with a 5 cm lens. After the lens, the light passed through a polarizer set to the magic angle. The light was then focused into a monochromator (HORIBA Jobin-Yvon; H-20) with a 10 cm lens. The detection wavelength was centered to 684, 715, or 720 nm. 684 nm was chosen to look at fluorescence emanating from the Qy band of chlorophyll a, and 715 and 720 nm were chosen to mimic the spectrum of light collected by Pulse Amplitude Modulated (PAM) fluorometers. The light from the monochromator was collimated with a 5 cm lens before being sent to the detector. The detector (Hamamatsu; R3809U) was cooled to -30°C, and computer-controlled (Becker and Hickl; DCC-100). The gain was set to 94%. At this gain, the instrument response function was 35-39 ps FWHM.

Using the method developed by Amarnath et al., the sequence of shutters opening and closing was designed in MATLAB and executed in LabVIEW [68]. The sequence of light exposure and detection due to the shutters could be arbitrarily designed. A typical measurement sequence consisted of an initial measurement taken on dark-adapted leaf. After two minutes, the leaves were illuminated with actinic light. After 10 minutes of illumination, the actinic light was blocked, and the leaf was allowed to relax for 10 minutes. Data was collected at intervals ranging from 10 to 60 seconds.
Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

2.3. Methods

Figure 2.1: A top-view schematic of the apparatus (a.) and detection geometry (b.). A 532 nm diode laser pumps an ultrafast Ti:Sapphire oscillator. The resulting 840 nm light is frequency doubled to 420 nm by a \(\beta\)-barium borate (BBO) crystal. After being doubled, the light is split by a beamsplitter. Part of the beam goes to a photodiode (PD) where it is used as a SYNC pulse for the time-correlated single photon counting (TCSPC) card. The other fraction of the beam is sent through a shutter to the leaf in its holder (green line). An actinic light is also sent through a shutter such that both beams can impinge on the leaf. After the leaf, the fluorescence is collected with a lens. It then passes through a polarizer set to the magic angle (Pol), variable neutral density (ND), and then it is focused into the monochromator for wavelength selectivity. After the monochromator, the light is collimated. It then passes through another shutter to the detector, a hybrid multichannel plate-photomultiplier tube (MCP-PMT). The resulting signal is sent to a detector control module (DCC) card and to the TCSPC card. The illumination and detection geometry is shown more detail in b. The leaf is held at a 45° angle to the actinic light and the detector. The angle between the laser and actinic light is 25°.

Unless otherwise specified, identical data collection routines were performed on multiple leaves from multiple plants (6 leaves total from 3 different plants). The data from each leaf were aligned according to the maximum of a cross-correlation (xcorAlign.m [74]). The curves were fit to a sum of exponentials (Picoquant Fluofit Pro 4.5). Curves were each fit to three decay functions and one fast rise (1-4 ps), as described by Eq. 3.3.2.1. This fast rise is below the instrument response function of the detector, but is needed in order to appropriately fit the decays (see Supplement for a comparison of the fit with 3 decays versus 3 decays and 1 rise in Fig. 2.10). Following data fitting, curves were reconstructed by plotting the fluorescence intensity calculated by the exponential decays:
2.4 Results

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

\[ F(t) = \sum_i A_i e^{-t/\tau_i} \quad \text{(2.3.0.1)} \]

Where \( A_i \) are the amplitudes and \( \tau_i \) are the fluorescence lifetime components. Amplitude-weighted average lifetimes of the leaves were calculated as follows:

\[ \tau_{\text{avg}} = \sum_i A_i \tau_i \quad \text{(2.3.0.2)} \]

The standard deviations of the values of \( \tau_{\text{avg}} \) were calculated by performing bootstrapping on the lifetimes. Bootstrapping involves selecting a subset of points in the fluorescence decay and determining the fluorescence lifetime components required to fit the subset of points. Repeating this process one thousand times gives an estimate of the error in the fluorescence lifetime fitting parameters. The amplitudes were bootstrapped separately to determine the error in the amplitudes of each component.

2.4 Results

2.4.1 Challenges to accurate fluorescence lifetime measurements

Pulse amplitude modulated (PAM) fluorometers are commonly used to measure the chlorophyll fluorescence yield and capture the dynamics of NPQ turning on and off. To calculate the amount of NPQ, the fluorescence yield is measured when the PSII reaction centers are closed with a saturating pulse of light [75]. Closing the PSII reaction centers ensures that any change in the fluorescence yield is due to a change in the amount NPQ rather than a change in photochemistry. Fluorescence yield measurements can easily determine when PSII reaction centers are closed because the fluorescence yield reaches a peak at this time. An example of a PAM measurement and the resulting NPQ curve are shown in Supplemental Fig. 2.11a. and Supplemental Fig. 2.11b., respectively, and the inset in Supplemental Fig. 2.11a. shows how the fluorescence yield changes during a 1 second exposure to saturating light to close the reaction centers for a dark-adapted leaf. For the fluorescence lifetime measurements to track changes due only to NPQ, it is necessary to measure fluorescence lifetimes when PSII reaction centers are closed. Because the fluorescence lifetime is proportional to the fluorescence yield, the fluorescence lifetime will also reach a peak value when the reaction centers are closed. One challenge, however, is that the amount of laser exposure needed to close the PSII reaction centers cannot be set at a constant value while a plant turns on and off NPQ because the amount of exposure to a saturating light required to close all the PSII reaction centers changes throughout quenching [71].

Just as PAM fluorometers make multiple fluorescence yield measurements during the saturating pulse to find the maximum, it is necessary to make multiple fluorescence lifetime measurements while the leaf is exposed to the saturating light to choose the measurement with highest fluorescence lifetime. Fig. 2.2b. illustrates how the fluorescence lifetime changes as a function of exposure time to the laser. 715 nm fluorescence was collected for 7 different
intervals of 0.2 seconds each, on 5 dark-adapted leaves per interval. Each interval corresponds to a different amount of laser exposure before the measurement, ranging from 0 to 0.6 seconds before the measurement. The leaves reach a peak average fluorescence lifetime after 0.4 seconds of laser exposure.

Fig. 2.2b. shows overlapping measurement intervals because different leaves were used for each measurement. However, for a sequence of fluorescence lifetime measurements during the induction and relaxation of NPQ, the fluorescence from each measurement on leaf is divided into different steps. This is shown in Fig. 2.2a. During a measurement, the leaf is exposed to the saturating light for 1 second. The total laser exposure time of 1 second was chosen because Kautsky curves have shown that leaves reach peak fluorescence in hundreds of milliseconds for saturating pulses with similar intensities [75, 76]. The fluorescence collected during this 1 second of exposure is separated into 5 steps of 0.2 seconds. Each step has a different amount of total laser exposure time, ranging from 0.2 seconds to 1 second. This is shown in Fig. 2.2a. The laser exposure time before the data is collected is referred to as the saturation time, because it is the amount of time that it takes to close all the reaction centers. The data for each step of each measurement on every individual leaf is aligned and summed. Then, each step of each measurement is fit. The step for each measurement with the largest value $\tau_{\text{avg}}$ is then used for further data analysis and all other steps are discarded, such that the end data set consists of one step per measurement.

Another challenge to measuring fluorescence lifetimes in leaves is that the high concentration of chloroplasts leads to reabsorption and subsequent re-emission of fluorescence. This has a large impact on the emission spectrum of leaves, which shows a peak at 735 nm that is absent in measurements on isolated chloroplasts [77]. It has been hypothesized that the re-emitted fluorescence prevents the accurate measurement of fluorescence lifetimes [69]. While relative changes in the fluorescence lifetime during quenching would still be instructive, for the measurements to be used to parameterize models, the fluorescence lifetimes need to be accurate. To verify the role of chlorophyll concentration on the measured fluorescence lifetime, the fluorescence lifetimes were compared between spinach leaves and solutions of spinach chloroplasts with varying concentrations at two different wavelengths, 684 nm and 720 nm. These wavelengths were chosen to correspond to fluorescence from the Qy band of chlorophyll (684 nm) and to a wavelength range closer to that measured in a PAM fluorometer (720 nm). It is expected that the average fluorescence lifetimes at 684 nm and 720 nm will differ because at each wavelength, there are different fractions of fluorescence collected from PSII and PSI antennas [78, 79], each of which exhibit different chlorophyll energy transfer dynamics. The average fluorescence lifetimes are shown in Table 2.1.

<table>
<thead>
<tr>
<th>[Chl] ($\mug/\muL$)</th>
<th>$\tau_{\text{avg}}$ (ns) at 684 nm</th>
<th>$\tau_{\text{avg}}$ (ns) at 720 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0025</td>
<td>1.20 ± 0.06</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>0.025</td>
<td>1.37 ± 0.07</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>0.125</td>
<td>1.44 ± 0.08</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>leaf</td>
<td>1.44 ± 0.07</td>
<td>0.51 ± 0.02</td>
</tr>
</tbody>
</table>

Table 2.1: Average fluorescence lifetimes of a spinach leaf and of spinach chloroplasts, measured at 684 nm and 720 nm.
2.4 Results

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

Figure 2.2: Multiple fluorescence measurements are required to determine the maximum average lifetime. A schematic for how a single measurement period is subdivided is shown in a. The measurement period is 1 second long, during which time the leaf is exposed to the laser and fluorescence is detected. The measurement period is subdivided into 5 steps, each with a 0.2 second duration. By fitting the data for each step, the average fluorescence lifetime can then be calculated for each step. The time with the maximum average lifetime (shown in gray, with the label peak \( \tau_{\text{avg}} \)) is the only step that is retained. The amount of laser exposure prior to the step with the maximum average fluorescence lifetime is referred to as the saturation time, because it is the time required to close all the PSII reaction centers. Measurements of \( \tau_{\text{avg}} \) for dark-adapted leaves are shown in b. The results are summed from 5 leaves. The fluorescence lifetimes are measured at 715 nm. Exposure to the laser begins at 0 seconds. The horizontal bars indicate the time span over which the measurement was made. The vertical bars indicate the standard deviation in the average fluorescence lifetime value. Because the maximum average fluorescence lifetime is from 0.3 seconds to 0.5 seconds, the saturation time for this measurement is 0.3 seconds.
The spinach leaf and chloroplast solutions with a concentration of chlorophyll of 0.025 and 0.125 $\mu$g $\mu$L$^{-1}$ all had nearly equivalent fluorescence lifetimes at 684 nm. At 720 nm, the spinach leaf has a fluorescence lifetime most similar to the lowest concentration of chloroplasts, 0.0025 $\mu$g $\mu$L$^{-1}$. Given that a typical spinach leaf has a chlorophyll concentration of approximately 2.7 $\mu$g $\mu$L$^{-1}$ [80, 81], the average fluorescence lifetime does show a concentration dependence over 3-4 magnitudes of chlorophyll concentration. The very dilute sample of chloroplasts (0.0025 $\mu$g $\mu$L$^{-1}$) displayed a lower average fluorescence lifetime. This could have been due to contamination of the sample in the cuvette which could overwhelm the fluorescence lifetime of chlorophyll at low chloroplast concentrations. The consistency of the fluorescence lifetimes between isolated chloroplasts and leaves indicates that the fluorescence lifetimes that we measure are accurate and uncontaminated by non-primary fluorescence. The lack of any concentration-dependence on the chlorophyll fluorescence lifetime is most likely because of a lack of time-dependence on the re-absorption of chlorophyll fluorescence. For a given wavelength, any fluorescent photon has an equal probability of being absorbed and re-emitted. The re-emission of the fluorescence would likely occur at longer wavelengths (due to the Stokes shift), and would be most apparent at 735 nm, where leaves display a secondary fluorescence peak due to re-absorption of fluorescence [77].

### 2.4.2 Applications of fluorescence lifetime measurements

To examine the effect of the actinic light intensity on the measured fluorescence lifetime, two actinic light intensities were selected that are typically used in NPQ measurements: 500 and 1200 $\mu$mol photons m$^{-2}$ s$^{-1}$. Lower actinic light intensities are not high enough to sustain NPQ [82], whereas higher light intensities can cause significant photodamage [83]. When the fluorescence lifetimes are measured throughout the induction and relaxation of NPQ, it is possible to see the fluorescence lifetimes as a function of time during or after exposure to actinic light. The series of measurements can therefore be visualized in a two-dimensional plot of the fluorescence intensity as a function of time following excitation and as a function of the illumination time. Plots for leaves illuminated with 500 and 1200 $\mu$mol photons m$^{-2}$ s$^{-1}$ light are shown in Fig. 2.3 a. and b, respectively. The normalized fluorescence intensity is calculated from the the exponential decays used to fit each fluorescence lifetime measurement, and is displayed on a log scale. The horizontal axis is the timescale of the fluorescence decay, which is in nanoseconds. The vertical axis is the timescale of light acclimation, which is minutes. The actinic light is on for 10 minutes, as shown by the white bar in Fig. 2.3.

The different dynamics for the induction of NPQ under two different illumination intensities can be seen by comparing the fluorescence intensity in the 2D plots. The leaves illuminated with 500 $\mu$mol photons m$^{-2}$ s$^{-1}$ have an initial shortening of the fluorescence lifetime during the first minute of illumination. Then, over the next two minutes, the decay lengthens. In minutes 3-5, the fluorescence lifetime shortens again, before stabilizing during the last 4 minutes of illumination. The dynamics of of the induction of quenching is different for the leaves illuminated with 1200 $\mu$mol photons m$^{-2}$ s$^{-1}$. Similar to the 500 $\mu$mol photons m$^{-2}$ s$^{-1}$ illumination leaves there is an initial shortening of the fluorescence lifetime during the first minute of light acclimation. However, after this time, the fluorescence
2.4. Results

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

Figure 2.3: Multiple measurements of the chlorophyll fluorescence lifetime for leaves illuminated with 500 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (a.) and 1200 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (b.) are represented in two-dimensional plots. These plots show how the fluorescence lifetime changes as a function of time following illumination with actinic light. A horizontal slice corresponds to the normalized fluorescence decay for a single measurement. A vertical slice shows how the percentage of excited chlorophyll at a particular time after excitation changes as a function of actinic light illumination. The fluorescence intensity is calculated from the fits to each fluorescence lifetime measurement. The maximum fluorescence for each measurement is normalized to 1. The fluorescence intensity is shown on a logarithmic scale. The decay time in nanoseconds is shown on the horizontal axis. The vertical axis is the time in minutes for the leaves’ acclimation to light. The actinic light turns on at 0 minutes, and is on until 10 minutes, at which point it is turned off for the remaining 10 minutes. The bars at the left of a. indicate when the actinic light is on (white) and off (black).

Integrating over the fluorescence decay axis results in the amplitude-weighted average fluorescence lifetime, shown in Fig. 2.4. Because the average fluorescence lifetime is proportional to the fluorescence yield, the dynamics of the average lifetime should resemble the dynamics of the fluorescence yield under conditions of closed PSII reaction centers measured with PAM fluorometers. However, unlike chlorophyll fluorescence yield measurements, the amplitude-weighted average fluorescence lifetime is not influenced by non-quenching processes such as chloroplast shielding and photobleaching. To assess how much non-quenching processes influence the amount of NPQ measured by PAM fluorometers, we compared the NPQ dynamics from the fluorescence lifetime measurements with the NPQ dynamics as measured through PAM fluorometry. Because the actinic light color has been shown to influence the chloroplast shielding, and in turn, the chlorophyll fluorescence yield, two different PAM fluorometers were used, each with a different color of actinic light. One uses a white actinic light (Hansatech FMS) [84], and the other uses a red actinic light (Walz Dual PAM) [85]. Both PAM fluorometers detect at similar wavelengths, >705 nm for the white light PAM, and >700 nm for the red light PAM.
nm for the red light PAM. The two different PAM fluorometers were both set to an actinic light intensity of 1000 $\mu$mol photons m\(^{-2}\) s\(^{-1}\). These illumination and detection parameters differ slightly from the fluorescence lifetime apparatus, which detects fluorescence centered at 684 nm and uses a white actinic light with an intensity of 1200 $\mu$mol photons m\(^{-2}\) s\(^{-1}\), though this difference in intensity is not expected to have a significant impact on the amount of quenching [86].

![Figure 2.4](image.png)

**Figure 2.4:** The average fluorescence lifetime for leaves illuminated with 500 $\mu$mol photons m\(^{-2}\) s\(^{-1}\) (gray) and 1200 $\mu$mol photons m\(^{-2}\) s\(^{-1}\) (black). The bars at the top of the figures indicate when the actinic light is on (white) and off (black).

In addition to calculating the standard NPQ parameter, we also calculated the quantum yield of NPQ with PSII reaction centers closed, $\phi_{NPQ,RCC}$. This term was chosen because it also reports the amount of quenching but, unlike the NPQ parameter, is linearly related to the fluorescence yield in the light and equal to the sum of the quantum yields of each component of NPQ when reaction center are closed [87].

The NPQ parameter from the PAM fluorescence measurements was calculated by comparing the fluorescence yield in the dark when PSII reaction centers are closed, $F_m$, and the fluorescence yield after light exposure when PSII reaction centers are closed, $F'_m$:

$$NPQ = \frac{F_m - F'_m}{F'_m}$$

(2.4.2.1)
2.4. Results

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

The NPQ curves from the fluorescence lifetime measurements were calculated using an analogous equation (see Supplement for derivation):

\[ NPQ = \frac{\tau_{\text{avg,dark}} - \tau_{\text{avg,light}}}{\tau_{\text{avg,light}}} \]  

(2.4.2.2)

The NPQ comparison between the fluorescence lifetime apparatus and the two PAM fluorometers is shown in Fig. 2.5a. For the values of \( \phi_{NPQ,RCC} \) from the PAM fluorescence measurements, the following equation was used:

\[ \phi_{NPQ,RCC} = \frac{F_m - F'_m}{F_m} \]  

(2.4.2.3)

The calculation of \( \phi_{NPQ,RCC} \) for the fluorescence lifetime measurements was adapted from the fluorescence yield measurements in the same manner as the NPQ parameter:

\[ \phi_{NPQ,RCC} = \frac{\tau_{\text{avg,dark}} - \tau_{\text{avg,light}}}{\tau_{\text{avg,dark}}} \]  

(2.4.2.4)

The comparison of \( \phi_{NPQ,RCC} \) between the fluorescence lifetime apparatus and the two PAM fluorometers is shown in Fig. 2.5b.

![Figure 2.5](image)

\( a. \) and \( b. \) from fluorescence lifetime measurements (black), a red (620 nm) actinic light PAM (Walz Dual PAM) (red), and a white actinic light PAM (Hansatech FMS) (green). The fluorescence lifetime measurements has a white actinic light with an intensity of 1200 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) and the PAM measurements have an actinic light intensity of 1000 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). The data points are shown as circles. The smoothed data is shown as lines. The bars at the top of the figures indicate when the actinic light is on (white) and off (black). Error bars corresponding to one standard deviation are shown for the NPQ PAM measurements.

The NPQ dynamics shown in Fig. 2.5a. during the induction of quenching are similar for the fluorescence lifetime measurement and the red actinic light PAM measurement. The two reach similar maximum values of NPQ as well. The NPQ curve from the white actinic light...
PAM measurement displays an additional rise component and reaches a maximum value of over 3, whereas the other two techniques reach a maximum value just under 2. However, the relaxation of NPQ is nearly identical for both the fluorescence lifetime measurement and the white actinic light PAM measurement, whereas the relaxation kinetics from the red actinic light PAM measurements are faster. The similarity between the amount of quenching measured by red actinic light PAM fluorometers and the fluorescence lifetime measurements suggests that the chlorophyll fluorescence yield NPQ parameter from red actinic light PAM fluorometers is uncontaminated by non-quenching processes such as photobleaching or chloroplast avoidance. As suggested by Cazzaniga and co-workers, the extra NPQ seen in the white actinic light PAM measurements does not appear to be due to quenching [67]. While both the white actinic light PAM and the fluorescence lifetime measurements show a slower relaxation of quenching than the red actinic light PAM, they most likely have a different origin. The slower relaxation in white light PAM measurements has been shown to be due to a reversal of chloroplast shielding [67]. The slower reversal of the fluorescence lifetime in measurements compared to the red actinic light PAM fluorescence yield measurements must be due to a relaxation of quenching processes. This could be due to a different response to the 420 nm laser light used in measurements compared to the saturating pulses of red or white light used in PAM fluorometers.

The NPQ-related dynamics and values of all three techniques are much more similar when $\phi_{NPQ,RCC}$ is plotted instead of NPQ. The induction dynamics seen in the $\phi_{NPQ,RCC}$ curves for the fluorescence lifetime measurement and the red actinic light PAM measurement are still very similar, but the difference between these two techniques and the white actinic light PAM measurement is less drastic. Similar to the NPQ relaxation kinetics, the relaxation of $\phi_{NPQ,RCC}$ is nearly identical for both the fluorescence lifetime measurement and the white actinic light PAM measurement, with the red actinic light PAM measurement showing faster relaxation. Comparing $\phi_{NPQ,RCC}$ rather than NPQ shows that the interpretation of the amount of quenching can vary drastically depending on which parameter is used in calculations.

In addition to being able to distinguish photobleaching and quenching, measuring the chlorophyll fluorescence lifetime can be used to gain a greater understanding of the energy transfer dynamics during quenching. This is because the lifetime of excited chlorophyll is determined by the number of quenching sites, their proximity to chlorophyll, and their rate of quenching. One way that the fluorescence lifetime decays can be used is to examine how the energy transfer dynamics change as the plant turns NPQ on and off. By fitting the fluorescence decays to three exponential decays, it is possible to plot how the lifetime, $\tau$, or amplitude, $A$, changes as a function of illumination. Each of the fluorescence lifetime components are shown in Supplemental Fig. 2.9. Supplemental Fig. 2.9 a, b, and c. show the long ($\tau_1$), medium ($\tau_2$), and short ($\tau_3$) lifetime components for leaves illuminated with 500 and 1200 $\mu$mol photons-m$^{-2}$-s$^{-1}$ light and Supplemental Fig. 2.9 d., e., and f. show the amplitudes, $A_1$, $A_2$, and $A_3$ for each of those lifetime components. Interpreting the dynamics of the lifetime components is complicated because the parameters obtained for the decays do not necessarily represent individual physical processes or groups of chlorophyll. Therefore, despite using three exponential decay components to describe the fluorescence decays, it is impossible to know whether this corresponds to three or more processes. Additionally, it is
2.4. Results

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

Challenging to assign physical meaning to the exponential decays because the fit parameters are coupled, meaning that the value of one parameter impacts the values of the other parameters that are fit [88]. While the three different decay components cannot be uniquely assigned to a particular process or group of chlorophyll, they do represent a characteristic timescale of chlorophyll relaxation. The lifetime of chlorophyll with the longest fluorescence timescale, \( \tau_1 \), varies between 2.4 and 1.4 ns, depending on the amount of quenching. The middle-timescale lifetime component, \( \tau_2 \), also varies depending on the amount of quenching, and has values between 1.4 and 0.6 ns. The shortest timescale quenching, \( \tau_3 \), varies between 0.17 and 0.12 ps.

Despite not being able to assign the three decays to particular processes, the shape of the fluorescence decay described by these exponentials is useful. One application is to compare the shape of the fluorescence decay when the amount of quenching (given by the value of NPQ or \( \phi_{NPQ,RCC} \)) is equal or the average fluorescence lifetimes are equal. As an example of such a comparison, the fluorescence decays for leaves illuminated with 500 \( \mu \)mol photons\( \cdot \)m\(^{-2} \cdot \)s\(^{-1} \) light are shown in Fig. 2.6. The two decays that are shown are for leaves after 60 seconds of illumination (gray) and for leaves 10 seconds after the actinic light has turned off (red). Both of these time points have nearly identical average fluorescence lifetime (0.62 ± 0.03 ns for the leaves after 60 seconds of illumination and 0.63 ± 0.03 ns for the leaves after 10 seconds of darkness). Fig. 2.6 a. shows the two decays, normalized to an initial intensity of 1 on a logarithmic intensity scale. The lifetimes and amplitudes that fit the fluorescence decay data in Fig. 2.6a. are shown in Fig. 2.6b. and Fig. 2.6c., respectively. The similarity between the fluorescence decays after 60 seconds of illumination and 10 seconds of relaxation suggests that the chlorophyll relaxation dynamics are nearly identical at these times. This similarity as NPQ turns on and off, shows the reversibility of NPQ. This type of fluorescence lifetime decay comparison can also be used to compare the relaxation and energy transfer dynamics between different genotypes, growth conditions, or chemical treatments.
2.5 Discussion

The ability to measure fluorescence lifetimes on leaves while NPQ turns on and off is desirable because it correlates changes in the amount of quenching with changes in the chlorophyll excited state relaxation dynamics. However, there has not been definitive proof that it is possible to measure the chlorophyll fluorescence lifetimes in leaves without the absorption and subsequent re-emission of fluorescence altering the measurement. Table 2.1 shows that the high concentration of chloroplasts in leaves does not impact the fluorescence lifetime measurement, and that it is possible to accurately measure the chlorophyll fluorescence lifetimes of leaves.

Previously, there have been measurements of the fluorescence lifetime on leaves during steady states in which quenching is off and on [70]. Measuring the fluorescence lifetime during a steady state allows for a long fluorescence detection period, increasing the signal-to-noise ratio. To measure the fluorescence lifetimes in leaves while the amount of NPQ is changing, it is necessary to have fluorescence detection periods much shorter than the changes in the amount of NPQ, and short enough to detect changes in the chlorophyll fluorescence transient, as shown in Fig. 2.2. Having a smaller detection window means reducing the amount of fluorescence detected from a single leaf. By implementing the computer-controlled shutter technique used by Amarnath and co-workers as shown in Fig. 2.1, we are able to repeat measurements on many different leaves and sum the data. In addition to giving a higher signal-to-noise ratio, it effectively averages over variation between leaves.

One benefit of monitoring the amount of NPQ through amplitude-weighted average fluorescence lifetime measurements as opposed to fluorescence yield measurements is that non-quenching processes such as chloroplast shielding and photobleaching do not influence the amount of NPQ measured. The quenching specificity of fluorescence lifetime measurements

Figure 2.6: Fluorescence lifetime decays for plants illuminated at 500 µmol photons·m⁻²·s⁻¹ are shown in a. The decay shown in black is from leaves illuminated for 60 seconds. The decay shown in gray is from leaves after 10 seconds of relaxation following 10 minutes of illumination. The fluorescence intensity is calculated from the fits to each fluorescence lifetime measurement. The values for the three fluorescence lifetime components and their associated amplitudes are shown in b. and c for leaves illuminated for 60 seconds (gray) and leaves after 10 seconds of relaxation (red). Black error bars represent 1 standard deviation.
2.6 Conclusions

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements can be seen in Fig. 2.5. In the future, this technique can help determine the amount of quenching during illumination intensities which also cause high photobleaching.

Previously, it had been impossible to know whether two samples with the same amount of NPQ have similar chlorophyll relaxation dynamics. This is because the fluorescence yield or any parameter derived from the fluorescence yield, such as the NPQ value or \( \phi_{NPQ,RCC} \) value, cannot distinguish between different processes that lower the fluorescence yield, and cannot distinguish between different combinations of factors that affect the amount quenching. However, the form of the chlorophyll fluorescence lifetime decay is determined by all the factors that influence the relaxation dynamics of chlorophyll.

While the same set of relaxation pathways cannot give rise to two different forms of fluorescence lifetime decay, the inverse is not true: two different sets of relaxation pathways may give rise to the same fluorescence decay curves. Therefore, while one can unambiguously identify when fluorescence lifetime decays corresponding to the same amount of quenching have different energy transfer dynamics, one cannot unambiguously determine when the dynamics of energy transfer are identical in two fluorescence lifetime measurements. The most that can be said for these cases is that, within the error of the measurement and data analysis, there is no sensitivity to a difference in the excited state dynamics of chlorophyll.

One application of this technique is to examine how these fluorescence decay comparisons can be used with mutants that lack a component known to contribute to NPQ. While it is common practice to assume that the difference in quenching between wild-type and a mutant is equivalent to the role of the mutation, this assumption is only valid in the case that the quenching that is left in the mutant is the same as the type of quenching in wild-type. For instance, the role of the protein, PsbS, is often defined as the NPQ from wild-type minus the amount of NPQ from npq4, a mutant that lacks PsbS [16]. By examining the shape of the fluorescence decays in wild-type and npq4 leaves when the amounts of NPQ are equal, it will be possible to determine whether the presence of PsbS significantly changes the dynamics of chlorophyll relaxation.

In addition to comparisons between fluorescence lifetime measurements, the fluorescence lifetime measurements can be used to benchmark energy transfer models. Because the fluorescence lifetime apparatus gives information not only on the nanosecond timescale of chlorophyll relaxation but also on the seconds to minutes timescale of how the energy landscape changes during quenching, it generates the decays for intermediate quenching states in addition to steady states. This can help constrain models to be able to follow how the energy transfer pathways change during the induction and relaxation of quenching.

2.6 Conclusions

We have developed a technique to measure fluorescence lifetime of chlorophyll in leaves throughout the induction and relaxation of NPQ. This is the first technique that is able to correlate the timescale over which NPQ changes with the timescale of excited state relaxation of chlorophyll. This technique gives a more specific measurement of quenching compared to fluorescence yield measurements, which is influenced by non-quenching processes that
alter the fluorescence yield. This technique also offers the ability to understand how NPQ modifies energy transfer and relaxation dynamics of chlorophyll in leaves. This technique will allow models of energy transfer and quenching to test against in vivo measurements of leaves in different quenching states, from dark-acclimated steady state measurements to quenched steady state measurements, as well as any intermediate states. In the future, this technique can be easily extended to work with various A.thaliana mutants, leaves from other plants species, vacuum-infiltrated leaves, and different illumination sequences.

2.7 Supplemental

To confirm that the leaf holder allows the leaf to experience the same quenching dynamics as a leaf attached to the plant, the fluorescence decays were compared for a leaf attached to a plant, a leaf in the holder with the stem in water, and a leaf in the holder without water under different illumination conditions. The fluorescence decays were measured for dark-adapted leaves, throughout a 10 minute illumination period, and throughout a 60 minute relaxation period. All three leaves showed identical decays for the dark-adapted state because the detached leaves had been detached immediately before the experiment started. This is shown in Supp. Fig. 2.8 a. During the light acclimation period, the leaf with the stem in water showed the same amount of quenching and the same fluorescence decay shape as a leaf still on a plant, but the leaf that was kept dry was not able to reach the same level of quenching. This is shown in Supp. Fig. 2.8b. Interestingly, all three leaves had identical fluorescence decays after an hour of relaxation in the dark following the 10 minute illumination period (shown in Supp. Fig. 2.8 c.). However, the leaf with the stem in water maintained the same texture as the leaf on the plant, while the leaf with stem kept dry had shriveled. Because of the leaf in the sample holder with water showed identical quenching to the leaf attached to a plant, the sample holder was used for all experiments.

2.7.1 Chloroplast isolation

Chloroplasts were isolated from dark-adapted spinach following the procedure described by Sjögren and Clarke [89]. The chloroplasts were placed in 2 mm pathlength cuvettes (Starna) with the same illumination and detection geometry as spinach leaves. To accumulate 1000 counts in the maximum bin, each collection period was separated by 2 minutes of darkness to allow the chloroplasts or leaf to re-adapt to the dark. Additionally, the samples were mixed before each measurement to ensure an even distribution of chloroplasts in the solution. The concentration of chlorophyll in chloroplasts was measured with a UV-VIS spectrophotometer, and the chloroplasts were examined under a microscope for intactness.

2.7.2 Effect of leaf age

To study whether the age of the plant impacts the average fluorescence lifetime, data were collected on 9 leaves from 6-week old plants and on 11 leaves from 8-week old plants. The
Figure 2.7: The device that hold the leaf in place consists of two parts: a clip and a holder. The leaf is clamped between two brass horseshoe-shaped pieces such that only the periphery of the leaf is held in contact with the brass. The tension that holds the leaf in position is due to a binder clip, which is attached to the top of each brass piece via epoxy. The leaf clip slides into a slot in the holder, which made out of Delrin. The leaf is positioned so that the stem dangles in the well, which is filled with 2 mL of water before each experiment. The water is changed every time a new leaf is put in the clip and holder.

Figure 2.8: The fluorescence decays for 3 leaves with different sample holder conditions and measured after 3 different illumination conditions are shown in a., b., and c. The image in a. shows the fluorescence decays for dark-adapted leaves. The image in b. shows the same leaves in a. after 10 minutes of illumination. The image in c. shows the same leaves as in b. after 60 minutes of relaxation in the dark. In each of the images, the fluorescence decays for the leaf attached to a plant are shown in black, the fluorescence decays for the leaf detached from the plant but with water are shown in gray, and the fluorescence decays for the leaf detached from the plant but without water are shown in red.

data were collected using a 48 nm detection bandwidth centered at 684 nm. While the average
fluorescence lifetime of the dark-adapted leaves was nearly identical, the steady-state average fluorescence lifetime during quenching (reached after 45 minutes of illumination with 500 µmol photons·m$^{-2}$·s$^{-1}$ light) differed by 80 picoseconds (see Tab. 2.2). This difference significantly affects the amount of NPQ.

<table>
<thead>
<tr>
<th>Age</th>
<th>$\tau_{\text{avg}}$ for dark-adapted (ns)</th>
<th>$\tau_{\text{avg}}$ for light-adapted (ns)</th>
<th>NPQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks old</td>
<td>1.37</td>
<td>0.52</td>
<td>1.64</td>
</tr>
<tr>
<td>8 weeks old</td>
<td>1.36</td>
<td>0.44</td>
<td>2.06</td>
</tr>
</tbody>
</table>

Table 2.2: The fluorescence lifetimes and resulting NPQ values from leaves of different ages

### 2.7.3 Derivation of NPQ term for fluorescence lifetimes

$F(t)$ is the chlorophyll fluorescence decay when PSII reaction centers are closed. Given the definitions of $F_m$ and $F_m'$, and Eqn 5.3.0.1, we retrieve the NPQ parameter as:

$$NPQ = \frac{\tau_{\text{avg,dark}} - \tau_{\text{avg,light}}}{\tau_{\text{avg,light}}}$$  (2.7.3.1)

### 2.7.4 Lifetimes and amplitudes

**Figure 2.9:** Fluorescence lifetime components and amplitudes for leaves illuminated with 500 µmol photons·m$^{-2}$·s$^{-1}$ light (gray) and 1200 µmol photons·m$^{-2}$·s$^{-1}$ light (black). The fluorescence lifetimes, $\tau_1$, $\tau_2$, and $\tau_3$, are shown in a., b., and c., respectively. The amplitudes $A_1$, $A_2$, and $A_3$ are shown in d., e., and f., respectively.
2.7. Supplemental

Characterizing non-photochemical quenching through time-resolved and steady-state fluorescence measurements

Figure 2.10: The instrument response function (IRF) and fluorescence decay data for a measurement on leaves illuminated with 500 µmol photons m\(^{-2}\)s\(^{-1}\) light for 1 minute are shown in a. and b. In a., the decay is fit to three exponential decays and in b., the decay is fit to three exponential decays and one exponential rise. The difference in the goodness of fit can be seen in the residuals, shown below the fluorescence decays in a. and b.
Figure 2.11: A PAM measurement from a leaf using a Hansatech FMS is shown in (a). The actinic light intensity used is $500 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The inset shows how the fluorescence yield changes during 1 second of illumination during the measurement of the maximum fluorescence yield in the dark ($F_\text{m}$). The NPQ curve generated from the PAM measurement is shown in (b).
Chapter 3

PsbS Catalyzes Energy-Dependent Quenching

3.1 Collaborators

The cross of npq4 and npq1 to create the npq4 npq1 mutant was done by Dr. Matthew Brooks. HPLC measurements and the growth of npq1 and npq1 npq4 plants were done by Dr. Alizée Malnoë. Eleonora De Re assisted with the data analysis. Alexandra Fischer assisted with data collection on the npq1 mutant. Robert Shih assisted in growing plants for experiments.

3.2 Introduction

Plants regulate light harvesting in Photosystem II (PSII) in response to changes in light intensity. One way that plants are able to regulate light harvesting is through turning on and off mechanisms that dissipate excess energy. These dissipation mechanisms are called non-photochemical quenching (NPQ). Energy-dependent quenching (qE) is the component of NPQ with the fastest reversibility. It turns on and off in seconds to minutes, and allows plants to adjust to fast intensity fluctuations due to intermittent shading. If plants are unable to turn on quenching quickly, the plant is not able to produce as many seeds or fruit presumably because of increased levels of photodamage [13]. If plants are unable to turn quenching off quickly, the plant dissipates energy that could be used in productive photosynthesis[14].

It is known that upon illumination with high light, a electrochemical gradient ($\Delta\psi$) and a pH-gradient ($\Delta$pH) form across the thylakoid membrane. The pH-gradient is thought to trigger different proteins and enzymes to induce quenching. In contrast, $\Delta\psi$ is not thought to contribute to triggering qE [90]. The components involved in qE that are pH-triggered include an enzyme, violaxanthin de-epoxidase (VDE)[91], and a small-transmembrane protein, PsbS.

The proteins and enzymes involved in NPQ can be identified through screening mutants for altered NPQ dynamics. The amount of NPQ is measured by a change in the chlorophyll fluorescence yield as a leaf is exposed to different light conditions. Mutants that display altered fluorescence yield dynamics can then be further examined to determine what change
is responsible for a different NPQ response. The mutant, \textit{npq1}, which lacks VDE and cannot convert violaxanthin to zeaxanthin has a phenotype with lower qE compared to wild-type [31]. Transient absorption measurements have found evidence of zeaxanthin cation formation during quenching, suggesting that energy dissipation during qE may occur through the formation of a chlorophyll-zeaxanthin heterodimer that undergoes charge separation followed by recombination [29]. The role of PsbS in quenching was also identified through changes in the fluorescence yield dynamics. The \textit{npq4} mutant, which lacks PsbS [92], shows no rapidly reversible quenching of chlorophyll fluorescence, which suggests that PsbS is necessary for qE \textit{in vivo} [35]. The \textit{npq4-E122Q} and \textit{npq4-E226Q} mutants, each of which has one lumen-exposed glutamate residue mutated such that it cannot be protonated, have qE levels that are midway between that of wild-type and \textit{npq4}. These findings show that PsbS is pH-sensitive and likely undergoes some conformational change when the lumen pH is low [36]. While mutant studies have shown that PsbS is necessary for fast, reversible quenching, it is still unclear what its precise role is. It was originally believed that PsbS might contain pigments that would directly quench excited chlorophyll [92, 35]. However, it was later shown that PsbS does not bind pigments, and thus cannot be the site where quenching occurs [93]. It has therefore been hypothesized that PsbS plays an indirect role in quenching, perhaps facilitating a rearrangement of proteins within the grana [33, 94]. However, because the fluorescence yield only gives information about the amount of quenching and does not contain information about the type of quenching, it cannot be used in isolation to understand what PsbS or any other protein or enzyme does to facilitate quenching.

One strategy to learn more about the role of PsbS has been to see what conditions can make \textit{npq4} plants quench as much as wild-type plants. By elevating the \(\Delta p\)H in isolated chloroplasts from wild-type and \textit{npq4}, Johnson and Ruban have been able to increase quenching in \textit{npq4}, suggesting PsbS may act as a catalyst for qE [95, 96]. One potential problem with these studies is the use of chemical inhibitors necessitates studying isolated chloroplasts rather than intact leaves. Additionally, the equivalent amounts of quenching still do not prove that the type of quenching in \textit{npq4} is the same as in wild-type. To try to understand how PsbS facilitates quenching, electron microscopy (EM) has been used to image thylakoid membranes acclimated to different light conditions [97] and with differing amounts of PsbS [98, 94]. The evidence has been conflicting. Freeze-fracture EM [94] and negative stain EM [98] on wild-type, \textit{npq4}, and a PsbS-over-expressor, \textit{L17}, show that the presence of PsbS reduces PSII crystalline array formation. However, the opposite effect of PsbS has been seen with negative stain EM on wild-type plants with different levels of PsbS expression due to different growth light intensities [97]. Recent work on proteoliposomes found evidence for the formation of a PsbS-LHCII heterodimer [99], suggesting that PsbS may have a direct role in LHCII rearrangement.

Fluorescence lifetime measurements, in contrast with fluorescence yield measurements, can be used to determine whether the relaxation dynamics of excited chlorophyll are modified by different mutations, informing on the role of a protein or enzyme during quenching. This is because the relaxation dynamics of excited chlorophyll during NPQ depends on many variables, including the distance to a quencher, the interactions between the orbitals of chlorophyll and the quencher, and the number of quenchers [100]. When many chlorophylls
are excited, as is the case during these measurements, there are many different relaxation rates contributing to the fluorescence decay. While different sets of processes may give rise to the same average fluorescence lifetime, they can give rise to different forms of the fluorescence lifetime decay. Therefore, the shape of the fluorescence lifetime decay curve can be used to determine whether two samples have similar excited chlorophyll relaxation dynamics. In this paper, we examine the fluorescence lifetime of chlorophyll throughout the induction and relaxation of quenching in leaves with and without PsbS and zeaxanthin to examine whether PsbS and zeaxanthin change the type of quenching occurring in plants. We show that the presence of PsbS does not change the chlorophyll relaxation dynamics for dark-adapted plants and during quenching, but that the \( npq1 \) mutation that prevents the synthesis of zeaxanthin drastically changes the excited state relaxation dynamics for chlorophyll, both in dark-adapted plants and during quenching. These measurements are performed in intact leaves without any chemical treatments, and strongly suggest that PsbS plays a catalytic role in vivo.

3.3 Methods

3.3.1 Plant material and growth conditions

Arabidopsis thaliana wild-type (ecotype Columbia 0) and mutant plants \( npq1 \) [31], \( npq4 \) [35] and \( npq1 npq4 \) [101] were grown on soil at a light intensity of 150 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \) under short day conditions (8 h light, 22C /16 h dark, 23C). 6 to 9 week-old plants were used for all experiments. Replicates were done using leaves from different plants grown at different times. \( A. \text{thaliana} \) plants were each dark-acclimatd for 30 minutes prior to detaching a leaf.

3.3.2 Fluorescence lifetime measurement

Experiments were conducted using a home-built fluorescence lifetime measurement apparatus described in Chapter 2. Similar to the set-up described by Amarnath et al., the light exposure of the leaf and detector were controlled by shutters programmed in LabVIEW. A 532 nm diode laser (Coherent G10) was used to pump a Ti:Sapphire oscillator (Coherent Mira 900) set to 840 nm. The resulting light was frequency-doubled to 420 nm with a \( \beta \)-barium borate (BBO) crystal to excite the Soret band of chlorophyll a (chl a). One portion of the light was directed to a photodiode (PD) to become the SYNC pulse for the time-correlated single photon counting (TCSPC) card (Becker-Hickl SPC-630 and SPC-850). The other portion of the laser light was intermittently blocked by a shutter. The average power of the laser at the sample was 5 mW with a pulse energy of 66 pJ. Detection of fluorescence was centered at 684 nm. In addition the laser light, an actinic light (Schott KL1500) with an intensity of 1200 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) was used to illuminate the sample. The leafs exposure to the actinic light was also controlled by a shutter. The dark-adapted leaves were exposed the actinic light for 45 minutes. After this first illumination, the actinic light was turned off for 3 minutes.
Then, the light was turned on for a second time for 10 minutes. The 65 fluorescence lifetime measurements were made at intervals ranging from 3 seconds to 60 seconds, depending on how fast the fluorescence lifetime was changing.

20 leaves from 6-8 plants were used for each genotype. The resulting data was aligned according to the maximum of a cross-correlation (xcorAlign.m [74]), and then summed. The fluorescence decay curves were fit to a sum of exponentials (Picoquant Fluofit Pro 4.5). Curves were each fit to three decay functions and one fast rise (1-4 ps), as described by Eq. 3.3.2.1. This fast rise was below the instrument response function of the detector, but was needed in order to appropriately fit the decays. Following data fitting, curves were reconstructed by plotting the fluorescence intensity calculated by the exponential decays:

\[
F(t) = \frac{\sum_i A_i e^{-t/\tau_i}}{\sum_i A_i}
\]  

(3.3.2.1)

Where \( A_i \) are the amplitudes and \( \tau_i \) are the fluorescence lifetime components. Amplitude-weighted average lifetimes of the leaves were calculated as follows:

\[
\tau_{avg} = \sum_i A_i \tau_i
\]  

(3.3.2.2)

The standard deviation of the values of \( \tau_{avg} \) were calculated by performing bootstrapping on the lifetimes. The amplitudes were bootstrapped separately to determine the error in the amplitudes of each component.

There were different procedures taken for measurements on the wild-type and npq4 leaves compared to the npq1 and npq1 npq4 leaves. Firstly, the plants were grown at different times and in different growth chambers. Secondly, the data were collected with different monochromators (a Horiba H20 with a groove grating density of 1200 gr/mm for the wild-type and npq4 leaves and a Horiba H20 with a groove density of 600 gr/mm for the npq1 and npq1 npq4 leaves). In the monochromator with the lower groove density, a 2 mm slit was used, allowing for a 16 nm bandwidth. In the monochromator with the higher groove density, no slit was used, allowing for a 48 nm bandwidth. Lastly, for the data collected on wild-type and npq4 leaves, the saturation times required to close the PSII reaction centers were determined by measuring the fluorescence lifetimes on 5 leaves with 1 second measurement times. The measurement times were broken into five 0.2 second steps. The fluorescence decays were then fit, and the laser saturation time to required to reach the highest value of \( \tau_{avg} \) was determined. Then, these timings were used to create the pulse sequence for 20 leaves. Due to redundancies in this method, for the npq1 and npq1 npq4 leaves, 20 leaves were measured with 1 second measurement times, with each of the measurement times broken into five 0.2 second steps. After fitting the decays for each step in the measurements, only the step with the longest value of \( \tau_{avg} \) was kept. To determine whether the difference in monochromator bandwidth or measurement method influences the fluorescence lifetime results, the fluorescence lifetimes of dark-adapted wild-type and npq4 leaves were compared using each of these two methods. The lifetime fits are shown in Supplemental Tab. 3.2. The measurement technique did not influence the lifetime measurement. This allows us to directly compare measurements taken with the 48 nm bandwidth with the data taken with 16 nm bandwidth.
3.3.3 Pigment Analysis

HPLC analysis of xanthophyll content was done as previously described [102]. A total of two samples from a set of plants grown at the same time were measured. Leaves were detached from several individual plants of same genotypes and exposed on moist filter paper to a light intensity of 500 $\mu$mol photons $\times$ m$^{-2} \times$ s$^{-1}$ for different given amount of time. Xanthophylls were quantified using standard curves of purified pigments and normalized to chlorophyll a.

3.4 Results

3.4.1 Average fluorescence lifetimes of wild-type and qE-mutants

To examine the dynamics of quenching, the fluorescence lifetimes were measured for wild-type, npq4, npq1, and npq1 npq4 leaves during a 45-minute illumination period. To deconvolute the dynamics of qE from longer-timescale NPQ mechanisms, the actinic light was turned off for 3 minutes. This amount of time is long enough to turn off the pH-gradient that triggers qE [103], but not long enough for significant conversion of zeaxanthin to violaxanthin [16]. The actinic light was then turned on for a 10 minute period to turn qE back on. In this manner, it is possible to examine the dynamics of qE in the absence and presence of other NPQ mechanisms. The average fluorescence lifetimes for wild-type, npq4, npq1, and npq1 npq4 over the duration of the experiments are shown in Fig. 3.1.

Both wild-type and npq4 leaves have nearly equal average fluorescence lifetimes in the dark. wild-type has a dark-adapted $\tau_{avg}$ of 1.36 ± 0.04 ns, and npq4 has a dark-adapted $\tau_{avg}$ value of 1.39 ± 0.04 ns. Both zeaxanthin-free mutants, npq1 and npq1 npq4, have nearly equal dark-adapted fluorescence lifetimes, as well, but these values are longer than wild-type and npq4. npq1 has a dark-adapted $\tau_{avg}$ of 1.51 ± 0.04 ns, and npq1 npq4 has a dark-adapted $\tau_{avg}$ value of 1.53 ± 0.04 ns.

When the actinic light was turned on, the average fluorescence lifetime decreased for wild-type and all of the mutants. However, the fluorescence lifetime for both plants with PsbS (wild-type and npq1) decreased much more rapidly than the mutants without PsbS (npq4 and npq1 npq4). While both wild-type and npq1 reached their steady-state value of quenching after 3-4 minutes of illumination, the fluorescence lifetimes for npq4 and npq1 npq4 decreased throughout the illumination period, though with progressively smaller changes in the fluorescence lifetime during the illumination period. After 30 minutes of illumination, npq4, npq1, and npq1 npq4 all reached approximately the same average fluorescence lifetime of 0.75 ns as compared to wild-type at this time, which had an average lifetime of 0.47 ns.

When the actinic light was turned off, the leaves that contain PsbS, wild-type and npq1, experienced an immediate increase in average fluorescence lifetime. However, both npq4 and npq1 npq4 experienced a transient decrease in the average fluorescence lifetime, dropping by approximately 30 ps. Then, the average fluorescence lifetime increased over the next two minutes of darkness. This is shown enlarged in the inset of Fig. 3.1.

When the actinic light was turned on for the second time, the average fluorescence lifetime of wild-type decreased by 40 ps within 3 seconds of illumination. However, the fluorescence
Figure 3.1: Average fluorescence lifetimes of wild-type (black), npq1 (blue), npq4 (red) and npq1 npq4 (purple) are shown as closed circles. The green open circles indicate the two similar average fluorescence lifetimes for wild-type and npq4 that are used in comparisons of the form of the fluorescence decays in Fig. 3.4. The orange open circles indicate the two similar average fluorescence lifetimes for npq1 and npq1 npq4 that are used in comparisons of the form of the fluorescence decays in Fig. 3.5. Circle 1. indicates a comparison between dark-adapted wild-type and npq4 leaves. Circle 1’. indicates a comparison between dark-adapted npq1 and npq1 npq4 leaves. Circles 2A. and 2B. indicate the comparison between wild-type leaves after 30 seconds of illumination and npq4 leaves after 30.5 minutes of illumination. Circle 2’. indicates the comparison between npq1 and npq1 npq4 leaves after 20.5 minutes of illumination. Circle 3. indicates a comparison between wild-type and npq4 during the second illumination period, after wild-type had been illuminated for 3 seconds and npq4 had been illuminated for 8 seconds. Circle 3’. indicates a comparison between npq1 and npq1 npq4 during the second illumination period, after 2.5 minutes of illumination. Top: actinic light on (white bar) and off (black bar). Inset: average fluorescence lifetimes during the light to dark to light transition, enlarged.

 lifetimes of npq1, npq4, and npq1 npq4 continued to increase. npq1 began to show a drop in average fluorescence lifetime after 8 seconds of illumination. The fluorescence lifetime of npq4 decreased after 13 seconds of illumination, and the fluorescence lifetime of npq1 npq4 decreased after 20 seconds of illumination. This is shown enlarged in the inset of Fig. 3.1. The fluorescence lifetimes of wild-type and npq1 reached their steady-state fluorescence lifetime values after 13-20 seconds of illumination, with wild-type reaching an average fluorescence lifetime of approximately 0.47 ns, and npq1 reaching an average fluorescence lifetime of 0.70
3.4. Results

PsbS Catalyzes Energy-Dependent Quenching

ns. The lifetimes of npq4 and npq1 npq4 decreased more slowly, reaching final values similar to that of npq1.

3.4.2 Differences in the average fluorescence lifetimes between pairs of plants

Plotting the difference between the average fluorescence lifetimes, $\Delta \tau_{\text{avg},a,b} (\tau_{\text{avg},a} - \tau_{\text{avg},b})$ can give insight into how different components influence the dynamics of NPQ. Both $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ and $\Delta \tau_{\text{avg},\text{npq}1,\text{npq}1 \text{npq}4}$ are shown in Figure 3.2. This plot shows the role of PsbS in quenching in the presence and absence of zeaxanthin.

![Figure 3.2: The effect of PsbS on the amount of quenching. $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ (black) and $\Delta \tau_{\text{avg},\text{npq}1,\text{npq}1 \text{npq}4}$ (red) are shown. $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ is calculated by subtracting the average fluorescence lifetime of npq4 from that of wild-type, and $\Delta \tau_{\text{avg},\text{npq}1,\text{npq}1 \text{npq}4}$ is calculated by subtracting the average fluorescence lifetime of npq1 npq4 from that of npq1. When the value of $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ or $\Delta \tau_{\text{avg},\text{npq}1,\text{npq}1 \text{npq}4}$ equals 0 (shown by the dashed line), it indicates no difference in average lifetime between the two genotypes. A negative value of $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ means that wild-type has a shorter average fluorescence lifetime, i.e. stronger quenching, than npq4, and a negative value of $\Delta \tau_{\text{avg},\text{npq}1,\text{npq}1 \text{npq}4}$ means that npq1 has stronger quenching than npq1 npq4.

The absolute value of $\Delta \tau_{\text{avg},\text{wt},\text{npq}4}$ increased over the first 1.5 minutes of illumination,
with the biggest difference occurring between 10 and 30 seconds of illumination. After 1.5 minutes of illumination, the $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$ decreased as the induction of quenching in wild-type slowed down and reached its steady-state level while $\text{npq}_4$ continued to experience an increase in quenching. This suggests that PsbS plays a major role only within the first minute of illumination. The decreasing effect of PsbS during the illumination period reflects the fact that, without PsbS, a level of quenching closer to that of wild-type can be gradually reached.

Analyzing $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$ during the relaxation of quenching from this data is difficult due to the limited number of data points in the dark. However, the value of $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$ became positive as wild-type relaxed more than $\text{npq}_4$. When the actinic light turned back on for the second illumination period, the difference between the average fluorescence lifetimes showed the same dynamics observed during the first illumination period, with the biggest change seen between 3 and 8 seconds of illumination. However, after 2.5 minutes of illumination, the difference between wild-type and $\text{npq}_4$ stabilized.

To further examine the PsbS-dependent relaxation kinetics, wild-type and $\text{npq}_4$ plants were illuminated for 10 minutes with higher light intensity (1200 $\mu$mol photons $\times$ m$^{-2} \times$ s$^{-1}$) and then allowed to relax for 10 minutes. The average fluorescence lifetimes are shown in Fig. 3.3a and the difference in average fluorescence lifetime is shown in Fig. 3.3b. With this higher actinic light intensity, both wild-type and $\text{npq}_4$ reached shorter lifetimes during quenching than when illuminated with 500 $\mu$mol photons $\times$ m$^{-2} \times$ s$^{-1}$ light, but the average lifetime of $\text{npq}_4$ was closer to that of wild-type. This manifests itself in a more reversible peak in Fig. 3.3b. The increase in quenching in $\text{npq}_4$ when the light was turned off can be seen clearly at the 10 minute mark. The analysis of $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$ during the relaxation is complicated by the fact that unlike during the induction of quenching, in which both wild-type and $\text{npq}_4$ displayed an increase in quenching, during the relaxation, wild-type experienced a biphasic decrease in quenching but $\text{npq}_4$ had a sudden increase in quenching during the first 30 seconds after the actinic light is turned off, followed by a linear decrease in quenching. After two minutes of darkness, wild-type and $\text{npq}_4$ recovered at the same rate. This shows that during recovery in the dark, unlike during illumination, PsbS only affects the quenching dynamics in the first minutes following the transition between light and dark.

To examine whether the presence of zeaxanthin changes the role of PsbS, the difference in average fluorescence lifetime was also calculated for $\text{npq}_1$ and $\text{npq}_1 \text{npq}_4$. This is shown in Fig. 3.2. Similar to $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$, during the first minutes of light, the dynamics $\Delta \tau_{\text{avg}, \text{npq}_1 \text{npq}_1 \text{npq}_4}$ showed a negative peak following the transition from dark to light. However, without zeaxanthin, the largest magnitude of $\tau_{\text{avg}, \text{diff}}$, shifted 30 seconds earlier, occurring after 1 minute of illumination. The magnitude of the lifetime difference was also slightly smaller. Additionally, the decrease in the magnitude of $\Delta \tau_{\text{avg}, \text{npq}_1 \text{npq}_1 \text{npq}_4}$ happened more quickly compared to $\Delta \tau_{\text{avg}, \text{wt}, \text{npq}_4}$. When the actinic light turned off, $\Delta \tau_{\text{avg}, \text{npq}_1 \text{npq}_1 \text{npq}_4}$ reached a steady state within a minute, indicating that both $\text{npq}_1$ and $\text{npq}_1 \text{npq}_4$ relax at the same rate.
3.4. Results

PsbS Catalyzes Energy-Dependent Quenching

Figure 3.3: The role of PsbS in plants illuminated with high light. The average fluorescence lifetimes of wild-type (black) and npq4 (red) during 10 minutes of illumination with 1200 µmol photons m⁻² s⁻¹ and 10 minutes of relaxation are shown in a. The difference between average fluorescence lifetimes of wild-type and npq4, Δτavg,wt,npq4, is shown in b.

3.4.3 Fluorescence decay comparisons

Fluorescence decays of wild-type and npq4

To examine whether the presence of PsbS impacts the relaxation dynamics of excited chlorophyll, the form of the fluorescence lifetime decays were compared between wild-type and npq4 at three different sets of points: on dark-adapted leaves before exposure to actinic light, on leaves with quenching on during the first illumination period, and on leaves with quenching on during the second illumination period. These comparison points are shown by green circles in Fig. 3.1.

The comparison between dark-adapted wild-type and npq4 leaves shows whether the presence of PsbS impacts the relaxation of excited chlorophyll when NPQ is off. The fluorescence decays, reconstructed by the fit to the data, are shown in Fig. 3.4a. The decay shapes are identical for wild-type and npq4. This was confirmed by examining the values for the fluorescence lifetime components and their associated amplitudes, shown in Supplemental Figure 3.7a. and Supplemental Figure 3.7b.

To examine whether the presence of PsbS impacts the relaxation dynamics of chlorophyll when NPQ is on, the fluorescence lifetime decays were compared between wild-type and npq4 at times during the induction of quenching in which they have the same average fluorescence lifetime. Both plants reached an average fluorescence lifetime of 0.77 ns during illumination (0.77 ± 0.03 ns for wild-type after 30 seconds of illumination and 0.77 ± 0.02 ns for npq4 after 31.5 minutes of illumination). The fluorescence decay shapes are shown in Fig. 3.4b. Despite equal values of average fluorescence lifetime, the fluorescence decays shapes were different for the two genotypes. The long decay component was shorter for wild-type compared to npq4 and the medium component was longer for wild-type compared to npq4, as shown in Supplemental Figure 3.7c. The amplitudes for each of the lifetime
components were similar for wild-type and \textit{npq4}, as shown in Supplemental Figure 3.7d.

In order to compare wild-type and \textit{npq4} with equal fluorescence lifetimes during quenching, it was necessary to compare the plants when they had experienced very different durations of illumination. It is known that the concentration of zeaxanthin changes during illumination on a timescale of minutes to tens of minutes [16]. Therefore, we hypothesized that the difference in fluorescence decay shapes is due to a difference in zeaxanthin concentration. To confirm this hypothesis, wild-type and \textit{npq4} leaves were illuminated with 500 \(\mu\)mol photons \(\times\) m\(^{-2}\) \(\times\) s\(^{-1}\) actinic light for 30 seconds and 30 minutes before extracting the pigments and taking HPLC measurements. The results of these measurements are shown in Table 3.1. There was no zeaxanthin detected in either wild-type or \textit{npq4} after 30 seconds of illumination. However, after 30 minutes of illumination, there was approximately 21 mmol of zeaxanthin per mole of chlorophyll a detected in wild-type, suggesting that the difference in fluorescence lifetime decay shape is due to a difference in zeaxanthin presence. In order to better determine whether PsbS changes the relaxation dynamics of chlorophyll, after 45 minutes of illumination, the actinic light was turned off for 3 minutes and then turned on again. This time period was chosen to relax qE-type quenching while maintaining a high concentration of zeaxanthin. The conversion of zeaxanthin to violaxanthin is much slower than 3 minutes, making it possible to compare wild-type and \textit{npq4} when zeaxanthin is present in both samples. During the second illumination period, wild-type and \textit{npq4} leaves reached equivalent values for the average fluorescence lifetime after 3 and 8 seconds of illumination, respectively. The fluorescence decays are shown in Fig. 3.4c. The fitting parameters are shown in Supplemental Figure 3.7e. and Supplemental Figure 3.7f. In this comparison, the average fluorescence lifetimes of wild-type and \textit{npq4} were identical again. The fluorescence lifetime decay components were also the same. While the amplitude components differ, the error in these terms is large and it does not seem to affect the fluorescence decay dynamics as indicated by the similarity of the decay shapes.
3.4. Results

Table 3.1: Pigment analysis of wild-type and npq4

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Light condition</th>
<th>Violaxanthin content (mmol per mole chl a)</th>
<th>Antheraxanthin content (mmol per mole chl a)</th>
<th>Zeaxanthin content (mmol per mole of chl a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dark-adapted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 seconds of light</td>
<td>48</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 minutes of light</td>
<td>51</td>
<td>9.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dark-adapted</td>
<td>54</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 seconds of light</td>
<td>43</td>
<td>7.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 minutes of light</td>
<td>20</td>
<td>13</td>
<td>21</td>
</tr>
</tbody>
</table>

Fluorescence decays of npq1 and npq1 npq4

If the difference in quenching shown in Fig. 3.4b. was due to a difference in the zeaxanthin concentration, it is expected that without zeaxanthin present, the presence of PsbS will not change the relaxation dynamics of excited chlorophyll during quenching. To test this hypothesis, fluorescence lifetimes were measured on npq1 and npq1 npq4. This is the equivalent of repeating the comparison between wild-type and npq4 in a zeaxanthin-free background. Due to the similarity of the average fluorescence lifetime traces between these two mutants, there were multiple points at which the average fluorescence lifetime curves for npq1 and npq1 npq4 crossed. The fluorescence decays at these crossing points are ideal for comparisons because it means that the two mutants have the same average fluorescence lifetime after having undergone the same amount of illumination. Thus the variation in concentration and other possible differences that could arise from different amounts of illumination are eliminated. We were able to make three comparisons: dark-adapted npq1 compared to dark-adapted npq1 npq4, npq1 and npq1 npq4 after 20.5 minutes of illumination, and npq1 and npq1 npq4 after 2.5 minutes of illumination in the second illumination period. These comparison points are shown by orange circles in Fig. 3.1.

The comparison between dark-acclimated npq1 and npq1 npq4 is shown in Fig. 3.5a. The fluorescence decay shapes were identical. The fitting parameters, shown in Supplemental Figure 3.8a. and Supplemental Figure 3.8b., were also identical within error. This result confirms that the presence of PsbS does not affect the relaxation dynamics of chlorophyll for dark-adapted leaves.

Next, the fluorescence lifetimes of light-adapted npq1 and npq1 npq4 were compared to examine how the presence of PsbS affects the dynamics of quenching when NPQ is on, but there is no zeaxanthin present. The fluorescence decays were compared where the average fluorescence lifetime curves cross at 20.5 minutes of illumination at an average fluorescence lifetime of 0.77 ns. The fluorescence decays for these two mutants are shown in Fig. 3.5b. Again, the decay curves were identical. This can also be seen in the fluorescence decay fit parameters shown in Supplemental Fig. 3.8c. and Supplemental Fig. 3.8d.

Lastly, the fluorescence decays for npq1 and npq1 npq4 were compared during the second illumination time after 2.5 minutes of illumination. At this time, the average fluorescence lifetimes were 0.69 for npq1 npq4 and 0.70 for npq1 npq4. The fluorescence decays for this
comparison are shown in Fig. 3.5c and are very similar. The fitting parameters are shown in Supplemental Figure 3.8e. and Supplemental Figure 3.8f.

In all three sets of comparisons, the fluorescence decays for both npq1 and npq1 npq4 are essentially identical, despite the difference in the presence or absence of PsbS. This indicates that the presence of PsbS does not significantly impact the relaxation of chlorophyll in the absence of zeaxanthin.

Because of the similarity of npq1 and npq1 npq4 after 20.5 minutes of illumination, of wild-type after 30 seconds of illumination, and of npq4 after 30.5 minutes of illumination, we compared the fit components to see if the presence of zeaxanthin or the ability to form zeaxanthin alters the fluorescence decay curve when the amount of quenching is the same. The lifetimes and amplitudes for all three mutants and wild-type with an average fluorescence lifetime of 0.77 ns are shown in Fig. 3.6a. and b., respectively. The lifetime and amplitude parameters for npq4 are slightly different than that of npq1 and npq1 npq4, though they are within error of one another. However, the lifetime parameters for wild-type are distinct from each of the qE mutants. In wild-type, the fluorescence lifetime of the longest component is shorter and the fluorescence lifetime of the medium lifetime component is longer compared to the qE mutants.
3.5 Discussion

Fluorescence lifetime decay similarity between dark-adapted wild-type and dark-adapted npq4 and between npq1 and npq1 npq4 shown in Fig. 3.4a. and Fig. 3.5a., respectively, confirms that the presence of PsbS does not influence the relaxation dynamics of chlorophyll when quenching is off. The difference in the form of the fluorescence decay between wild-type leaves that are illuminated for 30 seconds and for npq4 leaves that are illuminated for 30.5 minutes (shown in Fig. 3.4b.) may be due to a number of factors. However, the difference in zeaxanthin concentration is most likely a contributing factor because zeaxanthin is thought to play a significant role in energy transfer, either directly acting as a quencher [29] or indirectly by influencing the membrane morphology [104]. The difference in zeaxanthin concentration causing the difference in the fluorescence lifetime decays is confirmed both by the similarity between the fluorescence lifetime decays when both wild-type and npq4 have accumulated zeaxanthin (shown in Fig. 3.4c.) and during quenching for leaves that cannot convert violaxanthin to zeaxanthin (Fig. 3.5b. and Fig. 3.5c.). Because the fluorescence lifetime decays for plants with and without PsbS are identical when the average fluorescence lifetimes are the same (controlling the illumination time and zeaxanthin concentration), the presence of PsbS does not appear to change the relaxation dynamics of chlorophyll. This supports the hypothesis that PsbS acts as a catalyst to accelerate the induction of quenching, but does not itself affect the possible relaxation pathways.

In contrast to PsbS, the ability to convert violaxanthin to zeaxanthin does appear to strongly impact the dynamics of chlorophyll relaxation. Fig. 3.1 shows that dark-adapted npq1 and npq1 npq4 leaves have a longer average fluorescence lifetime than leaves that contain VDE. This is somewhat surprising since there is an undetectable amount of zeaxanthin
in wild-type leaves in the dark. However, it is possible that the lack of VDE in npq1 might somehow impact the relaxation dynamics of chlorophyll. A comparison between the fluorescence decays of wild-type and npq1 and npq1 npq4 when the fluorescence lifetimes are equal (shown in Fig. 3.6) confirms that the presence of zeaxanthin alters the chlorophyll relaxation dynamics. This supports the idea that the zeaxanthin serves a direct quenching role [16], perhaps, as suggested, through formation of a zeaxanthin cation [29].

Because we have shown that the presence of PsbS does not affect the relaxation dynamics of chlorophyll for equivalent zeaxanthin concentrations, it is possible to subtract the average fluorescence lifetime of npq4 from that of wild-type and to subtract the average lifetime of npq1 npq4 from npq1. While it is common to subtract NPQ curves to examine the role of a mutation, the NPQ term distorts the dynamics of quenching [105, 87], and therefore can change the interpretation of a mutation. In contrast, these subtractions reveal the amount of extra quenching that PsbS provides.

The large negative peak in the difference in average fluorescence lifetime $\Delta \tau_{avg,wt,npq4}$ in Fig. 3.2 and Fig. 3.3b., shows that the presence of PsbS has an increasing importance during the first minute of illumination. After one minute of illumination, the importance of PsbS lessens. This negative peak is narrower, more well-defined, and shifted to an even earlier time in $\Delta \tau_{avg,npq1,wt,npq4}$. The timing of the negative peak is surprising, because it suggests that the presence of PsbS is most important during the increase of the pH gradient rather than when the pH reaches its steady-state value, which occurs after 1 to 1.5 minutes of illumination [106, 96]. After 20.5 minutes of exposure to the actinic light, $\Delta \tau_{avg,npq1,wt,npq4}$ reaches a value of zero, showing that after 20.5 minutes of illumination, the presence of PsbS no longer increases the amount of quenching, despite the maintenance of a pH gradient. Because both $\Delta \tau_{avg,wt,npq4}$ and $\Delta \tau_{avg,npq1,wt,npq4}$ show the role of PsbS during the induction of quenching, the fact that the two curves have different dynamics indicates that the presence of zeaxanthin influences the role of PsbS. The fact that npq1, npq4, and npq1 npq4 all reach the same minimum value of average fluorescence lifetime after 45 minutes of illumination, as shown in Fig. 3.1 during quenching suggests that zeaxanthin and PsbS interact in wild-type leaves to cause a full quenching response, and the loss of either or both of these components has the same effect on the maximum amount of quenching possible.

During the second illumination period, only wild-type leaves display an immediate quenching response. npq4 and npq1 npq4, and to a lesser extent, npq1 have a delay between when the actinic light turns on and when quenching turns on (shown in the inset of Fig. 3.1). This indicates that some of the initial quenching seen in dark-adapted plants may be a combination of the induction of qE and the induction of downstream photochemistry due to the presence of actinic light. The delay in the quenching response of npq1 also indicates that an interaction between zeaxanthin and PsbS is responsible for strong quenching.

During the induction of quenching, both wild-type and npq4 display an increase in quenching, but during relaxation, wild-type and npq4 experience very different dynamics. wild-type leaves display a biphasic decrease in quenching but npq4 has a sudden increase in quenching during the first 30 seconds after the actinic light is turned off, followed by a linear decrease in quenching. After two minutes of darkness, wild-type and npq4 recover at the same rate. This indicates that only the first two minutes of relaxation are dependent on PsbS. It also shows
3.5. Discussion  

PsbS Catalyzes Energy-Dependent Quenching

that, without PsbS, quenching increases when the actinic light is turned off. The increase in quenching when PsbS is not present can be seen in the average lifetime of npq1 npq4 as well as in every other mutant that we have examined in the npq4 background such as soq1 npq4 (data not shown) [41]. This increase in quenching would not be expected if quenching was only sensitive to the ∆pH, because during this time period, ∆pH is decreasing towards zero [96, 106], and a lowered ∆pH corresponds to a lower amount of quenching [107]. However, during this time period, the electrochemical gradient, Δψ, reaches negative values, which are not experienced at any other times during quenching [108, 106]. This finding suggests that the increase in quenching could be due to Δψ.

Further evidence for Δψ influencing the quenching dynamics can be seen in the plots of Δτavg,wt,npq4 shown in Fig. 3.3b. The shape of Δτavg,wt,npq4 resembles the dynamics of -Δψ during the induction of quenching, but resembles the dynamics of ∆pH during the relaxation of quenching [108, 106]. Because Δτavg,wt,npq4 shows the difference in quenching due to PsbS, this suggests that the action of PsbS may be to respond to Δψ in addition to ΔpH. While studies have confirmed that mutating protonatable residues in PsbS results in the same phenotype as npq4 [36], protonatable residues are likely also responsive to positive ions other than protons. Therefore, any situations that would make PsbS unprotonatable would also destroy its responsiveness to other ions. We therefore hypothesize that initially, the interaction between PsbS and an excess of positive cations in the lumen causes the switch of PsbS to a quenching inducing state. As Δψ decreases and ∆pH increases, the interaction between PsbS and potassium and other cations is replaced by the increasing number of protons. When the actinic light is turned off, ∆pH decreases and Δψ becomes negative as protons move from the lumen to the stroma [109]. The resemblance of Δτavg,wt,npq4 to the ∆pH during the relaxation of quenching can therefore easily be explained by the fact that the ions that are moving are protons rather than other positive ions. When the actinic light is turned off, the increase in quenching when PsbS is absent and the fast decrease in quenching when PsbS is present, suggests that the decreases in ∆pH and Δψ cause PsbS to switch to a state that facilitates the relaxation of quenching.

In summary, our results show that while PsbS does not change the relaxation pathways available to chlorophyll, it serves three important roles. (1) It enables plants to sense changes in light intensity in under 30 seconds. (2) It protects plants from experiencing an increase in quenching when the light turns off. (3) PsbS, in concert with zeaxanthin, allows plants to reach a configuration in which many more chlorophylls have access to NPQ relaxation pathways. Interestingly, the quenching dynamics seen in plants without PsbS differ from qE mutants in other species. The npq4 mutant of C. reinhardtii, which lacks the protein, LhcSR, does not experience an increase in quenching when the actinic light turns off [110]. PsbS and LhcSR knockouts of the moss, Physcomitrella patens, also do not show an increase in quenching when the light turns off [111]. It has been suggested that the separation of qE from one protein that senses ∆pH and has quenchers into different proteins and processes, as is the case with plants, may help turn off quenching more rapidly [112]. Our results suggests that PsbS not only helps the plant respond quickly to changes in light intensity, but switches quenching on and off before the photosynthetic machinery would be able to do so otherwise. In this way, PsbS is able to robustly control the amount of quenching during
rapidly fluctuating light conditions.

### 3.6 Conclusions

The fluorescence lifetime measurements of wild-type, \( npq4 \), \( npq1 \), and \( npq1 npq4 \) plants give greater insight into how PsbS and zeaxanthin affect the amount and type of quenching. The absence of either zeaxanthin or PsbS, or the absence of both zeaxanthin and PsbS gives the same amount of quenching as measured by the value of average fluorescence lifetime after 45 minutes of illumination. We have also found that the action of PsbS occurs most strongly in the first minute of illumination and in the first two minutes of relaxation. While PsbS changes the amount of possible quenching, and the rate at which quenching turns on, it does not affect the relaxation dynamics of chlorophyll during quenching. In contrast, the ability to convert violaxanthin to zeaxanthin does change the relaxation dynamics of chlorophyll.

### 3.7 Supplemental

<table>
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<th>Parameter</th>
<th>wild-type (48 nm)</th>
<th>wild-type (16 nm)</th>
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<th>( npq4 ) (16 nm)</th>
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<tr>
<td>( \tau_{\text{avg}} ) (ns)</td>
<td>1.36 ± 0.04</td>
<td>1.33 ± 0.06</td>
<td>1.39 ± 0.04</td>
<td>1.44 ± 0.05</td>
</tr>
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<td>( \tau_1 )</td>
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<td>2.31 ± 0.05</td>
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<td>( \tau_2 )</td>
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</tr>
<tr>
<td>( \tau_3 )</td>
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Figure 3.7: The fitting parameters for fluorescence decay comparisons between wild-type (gray) and npq4 (red) are shown. The error bars reflect one standard deviation. a, c, and e show the values for the long, medium, and short fluorescence lifetime decay components for comparisons between wild-type and npq4, and b, d, and f show the associated amplitudes for the different fluorescence decay components. The comparison between fitting parameters for dark-adapted leaves are shown in a and b. The comparison between leaves during the first illumination period, after 30 seconds of illumination for wild-type and 30.5 minutes of illumination for wild-type, are shown in c and d. The comparison between leaves during the second illumination period, after 3 seconds of illumination for wild-type and 8 seconds of illumination for npq4 are shown in e and f.
Figure 3.8: The fitting parameters for fluorescence decay comparisons between *npq1* (gray) and *npq1 npq4* (red) are shown. The error bars reflect one standard deviation. *a*, *c*, and *e* show the values for the long, medium, and short fluorescence lifetime decay components for comparisons between *npq1* and *npq1 npq4*, and *b*, *d*, and *f* show the associated amplitudes for the different fluorescence decay components. The comparison between fitting parameters for dark-adapted leaves are shown in *a* and *b*. The comparison between leaves during the first illumination period, after 20.5 minutes of illumination, are shown in *c* and *d*. The comparison between leaves during the second illumination period, after 2.5 minutes of illumination are shown in *e* and *f*. 
Chapter 4

Characterizing a new type of NPQ mutant

4.1 Collaborators

The initial study of soq1 and wild-type was carried out in collaboration with Dr. Matthew Brooks in the lab of Professor Krishna Niyogi. Matthew Brooks isolated the soq1 mutant and performed the genetic analysis. He also grew the plants for the fluorescence lifetime experiments. Further experiments on the soq1 family of mutants, including soq1 npq4, D101, A164, as well as experiments with npq4 grown under different light conditions were carried out in collaboration with Dr. Alizée Malnoë in the lab of Professor Krishna Niyogi. Alizée Malnoë generated the family of soq1 suppressors and enhancers. In addition, she assisted with experimental design for the fluorescence lifetime experiments and grew plants for the experiments. Alexandra Fischer assisted with data collection on many of the soq1 mutants. Portions of this chapter have been adapted from Matthew D. Brooks, Emily J. Sylak-Glassman, Graham R. Fleming, and Krishna K. Niyogi “A thioredoxin-like/β-propeller protein maintains the efficiency of light harvesting in Arabidopsis.” Proceedings of the National Academy of Sciences, 110, no. 29 (2013): E2733-E2740.

4.2 Introduction

To discover the proteins, enzymes, and processes that regulate NPQ, random mutations in the Arabidopsis thaliana genome are introduced by exposing seeds to ethyl methanesulfonate (EMS). The seeds, grown to seedling stage, are then screened for enhanced or reduced quenching via chlorophyll fluorescence yield video imaging. Mutants with an interesting phenotype can then be further characterized to determine whether an NPQ-specific mutation has been generated. While a mutant can show an enhanced or reduced fluorescence yield, because the fluorescence yield is not an unambiguous measurement of NPQ, a mutant cannot be definitively identified as a quenching mutant by the fluorescence yield alone. In this chapter, we discuss the characterization of a new series of mutants to determine whether they are
true NPQ mutants, as well to investigate the quenching properties of these mutants.

### 4.2.1 Thioredoxins

Matthew Brooks discovered that plants that lack a particular thioredoxin protein show enhanced NPQ, both in a Columbia-0 (wild-type) background and in an npq4 background, as measured by pulse amplitude modulated (PAM) fluorescence. This protein, named SOQ1, for suppressor of quenching, shows enhanced quenching as measured through PAM fluorescence measurement [41]. Mutants lacking the SOQ1 protein are given the name, soq1.

Thioredoxins contain a conserved domain that includes four amino acids with a cysteine on either end, CXXC. These domains are often used in the reduction or oxidation of target proteins. Because mutations in the CXXC domain of SOQ1 affected the function of SOQ1 in preventing the additional quenching, it suggests that the role of SOQ1 is to regulate the oxidation or reduction of a target protein. A schematic is shown in Fig. 4.1. For a review, see [113].

![Figure 4.1: A thioredoxin acting on a target protein](image)

### 4.2.2 Previous experiments on soq1 mutants

Initial experiments showed that the extra quenching when SOQ1 is absent is not due to a change in photochemistry, suggesting that the extra quenching is due to NPQ [41]. Each known component of NPQ was altered in the absence of SOQ1 to see if the presence of SOQ1 regulates a known NPQ pathway.

Energy-dependent quenching (qE) is triggered by an increase in ΔpH. If soq1 achieves higher quenching through an increased ΔpH, it would be expected that destroying the ΔpH would eliminate the increased quenching. To test this hypothesis, leaves were vacuum-infiltrated with nigericin, a chemical that destroys the ΔpH gradient. While infiltration with nigericin lessened the amount of quenching in wild-type and soq1, the soq1 mutants still displayed higher quenching than wild-type, with the signature slower relaxation time. This indicates that the role of SOQ1 is unrelated to ΔpH and is not triggered by qE [41].

To test whether the increase in quenching is due to an increase in zeaxanthin, soq1 was crossed to the mutant npq1. The npq1 mutant lacks violaxanthin de-epoxidase (VDE), and cannot accumulate zeaxanthin. The soq1 npq1 mutant still shows enhanced quenching compared to npq1, indicating that the role of SOQ1 is unrelated to zeaxanthin. Similar tests were
4.3 Methods

Characterizing a new type of NPQ mutant

performed to test if the presence of SOQ1 affects state transitions. Crossing soq1 to stn7 plants, which cannot perform state transitions, does not eliminate the extra quenching [41].

It is believed that inhibition-quenching (qI) is due to damaged PSII complexes. If the action of SOQ1 is linked to qI, it is expected that inhibition of repair would further increase the amount of quenching. To test this, leaves were illuminated with high light to induce damage, in the presence and absence of lincomycin, a chemical inhibitor that prevents resynthesis of chloroplast-encoded proteins. However, the dynamics of soq1 are unchanged with lincomycin treatment [41].

4.2.3 Fluorescence lifetime measurements on soq1 mutants

The measurements on the soq1 mutants suggest a new form of regulated NPQ, however with fluorescence yield measurements, it is difficult to disambiguate photobleaching and quenching. To examine whether the lack of SOQ1 protein causes extra quenching, the fluorescence lifetimes were measured on wild-type and soq1. Additionally, npq4 and soq1 npq4 were compared to eliminate contributions of PsbS-dependent quenching. To try to see soq1-like quenching in plants that contain SOQ1, the fluorescence lifetimes of npq4 plants grown and illuminated with different actinic light intensities were measured. Lastly, a mutant that enhances the soq1 phenotype and has constitutive quenching was also examined.

4.3 Methods

Experiments were conducted using the apparatus and protocol described in Chapter 2 with the following modifications. After 30 minutes of dark-adaptation, leaves were exposed to actinic light for 10 minutes and then allowed to relax in the dark for 10 minutes. Measurements were taken as follows: the first measurement was taken on dark-adapted leaves. After 2 minutes, the actinic light was turned on. After 10 seconds, the first light-adapted measurement was taken. After another 20 seconds, another light-adapted measurement was taken. Additional measurements were taken every 30 seconds for the duration of the light period. After the actinic light was turned off, a measurement was made after 10 seconds. Another measurement was made after 20 seconds. The next measurement was made after 60 seconds. Additional measurements were made every 60 seconds until the plant had been in the dark for 9 minutes and 30 seconds. The last measurement was made after 10 minutes of dark adaptation. This measurement sequence was chosen in order to conduct measurements more frequently when the fluorescence lifetime changes the most.

The following plants were grown under 100 µmol photons × m⁻² × s⁻¹ light and illuminated with 1200 µmol photons × m⁻² × s⁻¹ actinic light: wild-type, soq1, npq4, soq1 npq4, A164, and D101. In addition, one group of npq4 plants was grown under 25 µmol photons × m⁻² × s⁻¹ light and illuminated with 2000 µmol photons × m⁻² × s⁻¹ actinic light. Twenty leaves of npq4 with each light condition and twenty leaves of soq1 npq4 were measured. 17 leaves of A164 were measured. 9 leaves of D101 were measured. 10 leaves of soq1 and 7 leaves of wild-type were measured.
There were different procedures taken for measurements on the \textit{soq1} and wild-type leaves compared to all of the other measurements made. Firstly, the plants were grown at different times and in different growth chambers. Secondly, the data were collected with different monochromators (a Horiba H20 with a groove grating density of 1200 gr/mm for the \textit{soq1} leaves and a Horiba H20 with a groove density of 600 gr/mm for all other leaves). In the monochromator with the lower groove density, a 2 mm slit was used, allowing for a 16 nm bandwidth. In the monochromator with the higher groove density, no slit was used, allowing for an approximately 48 nm bandwidth. Lastly, for the data collected on wild-type and \textit{soq1} leaves, the saturation times required to close the PSII reaction centers were determined by measuring the fluorescence lifetimes on 5 leaves with 1 second measurement times. The measurement times were broken into 5 0.2 second steps. The fluorescence decays were then fit, and the laser saturation time to required to reach the highest value of $\tau_{avg}$ was determined. Then, these timings were used to create the pulse sequence for 20 leaves. Due to redundancies in this method, for the \textit{npq4}, \textit{soq1 npq4}, and A164, 20 leaves were measured with 1 second measurement times, with each of the measurement times broken into 5 0.2 second steps. After fitting the decays for each step in each measurement, only the data set with the longest value of $\tau_{avg}$ was kept. To determine whether these measurement differences changes the fluorescence lifetime results, the fluorescence decays using both methods were repeated on dark-adapted wild-type leaves. The dark-adapted fluorescence lifetime values were similar, as shown in the Supplemental section of Chapter 3.

After measuring the fluorescence lifetimes for each of the mutants, the data was aligned, summed, and fit as described in Chapter 2.

### 4.4 Results

#### 4.4.1 Investigation of quenching in plants with and without SOQ1

To investigate whether the lowered fluorescence yield seen in PAM fluorescence measurements of the \textit{soq1} mutant is due to quenching or photobleaching, the fluorescence lifetimes were measured on \textit{soq1} and wild-type, and \textit{soq1 npq4} and \textit{npq4}. The comparison between wild-type and \textit{soq1} shows the role of SOQ1 in a background with PsbS, and the comparison between \textit{npq4} and \textit{soq1 npq4} shows the role of SOQ1 in a background without PsbS. The PsbS-free background was used because 10 minutes of 1200 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ causes significant amounts of PsbS-dependent quenching which can complicate the interpretation of the role of SOQ1.

The average fluorescence lifetimes for wild-type, \textit{soq1}, \textit{npq4}, and \textit{soq1 npq4} are shown in Fig. 4.2.

Upon illumination with actinic light, the average fluorescence lifetimes of wild-type and \textit{soq1} decreased strongly. The average fluorescence lifetimes of \textit{npq4} and \textit{soq1 npq4} also decreased, but not as rapidly as for the plants with PsbS. After 2.5 minutes of illumination, the average fluorescence lifetime of wild-type plateaued. However, the average fluorescence lifetimes of \textit{npq4}, \textit{soq1}, and \textit{soq1 npq4} continued to decrease throughout the illumination
4.4. Results

Characterizing a new type of NPQ mutant

Figure 4.2: The average fluorescence lifetimes of wild-type (black), soq1 (gray), npq4 (red), and soq1npq4 (blue).

Table 4.1: The biexponential decay parameters that fit the change in average fluorescence lifetimes during illumination of npq4 and soq1npq4 are shown below

<table>
<thead>
<tr>
<th>Mutant</th>
<th>rate$_1$ in min$^{-1}$ (lifetime$_1$ in min)</th>
<th>rate$_2$ in min$^{-1}$ (lifetime$_2$ in min)</th>
<th>R-square</th>
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<tbody>
<tr>
<td>npq4</td>
<td>0.67 (1.5)</td>
<td>0.023 (43)</td>
<td>0.9971</td>
</tr>
<tr>
<td>soq1 npq4</td>
<td>0.75 (1.3)</td>
<td>0.046 (22)</td>
<td>0.9977</td>
</tr>
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</table>

period. For all three mutants, the average fluorescence lifetime changed the most in the first minutes of illumination, with progressively smaller changes in the average fluorescence lifetime during the illumination period. The shape of the average fluorescence lifetime curve for all of these mutants was biphasic. For soq1, there was a fast decline in average fluorescence lifetime in the first 2.5 minutes, and then a slow linear decline in average fluorescence lifetime during the remaining illumination period. The decrease in the average lifetime for both npq4 and soq1npq4 were described well with a biexponential decays (R-Square >0.99). The fits to the data are shown in Supplemental Figure 4.17. The fit parameters are shown in Table 4.1. The two decay components for npq4 are 1.5 minutes and 43 minutes. The two decay components for soq1npq4 are 1.3 minutes and 22 minutes. This suggests that the timescale of longer decay component in npq4 depends on SOQ1 and that shorter decay is unaffected by SOQ1.

Ultimately, after 10 minutes of illumination, the average fluorescence lifetime of wild-
Characterizing a new type of NPQ mutant

4.4. Results

type leaves was 0.41 ns, the average fluorescence lifetime of soq1 leaves was 0.31 ns, the average fluorescence lifetime of npq4 leaves was 0.58 ns, and the average fluorescence lifetime of soq1 npq4 was 0.38 ns. This shows that the plants without SOQ1, soq1 and soq1 npq4, reach a more strongly quenching state than when SOQ1 is present.

When the actinic light was turned off, both wild-type and soq1 experienced a partial relaxation in the initial minutes of darkness. In the case of wild-type, the first, fast relaxation phase took place during the first 3 minutes after the actinic light turns off. In this period, the average lifetime climbed to 0.90 ns, where it remained for the duration of the relaxation period. Because this was not a complete recovery of the fluorescence lifetime, it is expected that the second phase of recovery is much slower. For soq1, when the actinic light was turned off, the average lifetime increased quickly, reaching 0.60 ns after 5 minutes of darkness. It remained at this value for the duration of the relaxation period. The dynamics for both mutants without PsbS were markedly different. When the actinic light was turned off, the average fluorescence lifetimes of both npq4 and soq1 npq4 decreased, though npq4 decreased more strongly than soq1 npq4. After 30 seconds, the average fluorescence lifetime increased. This increase in lifetime appeared linear, but fit well either linearly or with a monoexponential rise. When fit linearly, the slope of the recovery was faster in the case of npq4 compared to soq1 npq4 (21 ps per min for soq1 npq4 versus 30 ps per min for npq4). However, when fit to a mono-exponential rise, the timescale of recovery for npq4 was 22 minutes and the timescale for recovery for soq1 npq4 was 20 minutes. This suggests that the presence of SOQ1 does not significantly alter the speed with which quenching is turned off.

To understand how the presence of SOQ1 affects the excited state dynamics of chlorophyll for dark-adapted plants, the fluorescence decays were compared for wild-type, soq1, npq4, and soq1 npq4. The fluorescence decays for wild-type and soq1 are shown in Fig. 4.3a., and the fluorescence decays for npq4 and soq1 npq4 are shown in Fig. 4.3b. While the average fluorescence lifetime of soq1 is longer than wild-type (1.32 ns for wild-type versus 1.44 ns for soq1), this seems to be a fitting error because decay of soq1 is slightly shorter than that of wild-type. Similarly, the fluorescence decay of soq1 npq4 is slightly shorter than npq4, though this is reflected in the average fluorescence lifetime (1.37 ± 0.04 ns for soq1 npq4 and 1.44 ± 0.05 ns for npq4).

While the average fluorescence lifetimes show that the mutants without SOQ1 are able to quench more, we wanted to examine whether the type of quenching in soq1 mutants is different from the type of quenching when SOQ1 is present. To do this, the shape of the fluorescence decays was compared when the average fluorescence lifetimes are equal.

During the illumination period, the fluorescence decays of wild-type and soq1 are extremely similar when wild-type has experienced 6.5 minutes of illumination and when soq1 has experienced 2.5 minutes of illumination. This is shown in Fig. 4.4a. Additionally, the fluorescence lifetimes of npq4 and soq1 npq4 were similar for 3 minutes of illumination of npq4 and 1.5 minutes of illumination for soq1 npq4. Fig. 4.4b. Additionally, soq1 npq4 after 3 minutes of illumination and npq4 after 9.5 minutes of illumination are compared, as shown in Fig. 4.4c. At this time, too, the fluorescence decays are very similar, suggesting that the presence of SOQ1 regulates the amount of quenching, but does not allow or prevent a distinct quenching pathway.
4.4. Results  

Characterizing a new type of NPQ mutant

**Figure 4.3:** The fluorescence decays for dark-adapted wild-type (black) and *soq1* (red) are shown in *a*. The fluorescence decays for dark-adapted *npq4* (black) and *soq1 npq4* are shown in *b*.

**Figure 4.4:** The average fluorescence lifetimes of wild-type (black) after 6.5 minutes of illumination and of *soq1* (red) after 2.5 minutes of illumination are shown in *a*. The average fluorescence lifetimes of *npq4* (black) after 3 minutes of illumination and of *soq1 npq4* (red) after 1.5 minutes of illumination are shown in *b*. The average fluorescence lifetimes of *npq4* (black) after 9.5 minutes of illumination and of *soq1 npq4* (red) after 3 minutes of illumination are shown in *c*.

In the comparisons discussed above, the fluorescence decays are compared between plants without SOQ1 at early illumination times and plants with SOQ1 at later illumination times. This allows for comparisons between fluorescence decays when the average fluorescence lifetimes are equivalent, at these times. However, the presence of SOQ1 can be seen most strongly at later illumination times. Therefore, the comparisons at low quenching levels may not be representative of comparisons at high quenching. While there is no comparison available between high quenching with and without SOQ1, it is possible to investigate the quenching when SOQ1 is absent by comparing the fluorescence decays of a given mutant when the actinic light is off and on. Both *npq4* and *soq1 npq4* had the same average lifetimes after 7.5 minutes of illumination and 4.5 minutes of relaxation. In the case of *soq1 npq4*, the average fluorescence lifetime was 0.40 ns after for both of these measurements, and for *npq4*, the average fluorescence lifetime was 0.63 ns during illumination and 0.61 ns during relaxation. The fluorescence lifetime decays shapes for *npq4* and *soq1 npq4* are shown in...
Fig. 4.5a. and Fig. 4.5b., respectively. While the fluorescence decays for \(npq4\) are identical during illumination and relaxation, the fluorescence lifetimes for \(soq1 \ npq4\) show different decay dynamics. This suggests that the rate at which both \(soq1 \ npq4\) and \(npq4\) relax after illumination is unrelated to the presence of SOQ1. Because it has been shown that the extra quenching when SOQ1 is absent does relax on a timescale of hours [41], the similar rate of relaxation between \(soq1 \ npq4\) and \(npq4\) is because the extra quenching observed in \(soq1 \ npq4\) does not relax within the timescale of the experiment.

![Fluorescence decays for \(npq4\) and \(soq1 \ npq4\)](image)

**Figure 4.5:** The fluorescence decays for \(npq4\) and \(soq1 \ npq4\) are shown in a. and b. The two decays for each mutant are for 7.5 minutes of illumination (gray) and after 4.5 minutes of relaxation (red).

To test whether the presence of SOQ1 has any impact on the amount of photobleaching, the fluorescence decays for each leaf were summed for \(npq4\) and \(soq1 \ npq4\), and then divided by the number of leaves measured. This gives an estimate of the fluorescence decay for an average leaf. The maximum number of counts was compared for \(npq4\) and \(soq1 \ npq4\) for dark-adapted leaves and for leaves after 10 minutes of illumination. An average dark-adapted \(npq4\) leaves has a maximum fluorescence count of 314. This indicates 2% photobleaching. After 10 minutes of illumination, it drops slightly to 309 counts. \(soq1 \ npq4\) leaves have a maximum fluorescence count of 352 when dark-adapted. After 10 minutes of illumination, the maximum number of fluorescence counts drops to 335 counts. This indicates 5% photobleaching. This difference in the amount of photobleaching between \(soq1 \ npq4\) and \(npq4\) is much smaller than the leaf-to-leaf variation in the number of fluorescence counts, which indicates that it is likely not significant.

### 4.4.2 Investigating whether high light can induce enhanced quenching

The NPQ phenotype of plants, as measured by chlorophyll fluorescence yield, is highly dependent on the plants’ growth conditions and the actinic light illumination intensity. This can
be seen with soq1 mutants: the enhanced quenching seen in plants without SOQ1 can only be seen when the actinic light intensity is high [41]. Fig. 4.6 shows the amount of NPQ for npq4 and soq1 npq4 with different growth and illumination intensities. The soq1 npq4 leaves were grown in extremely bright light (2000 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \)), and illuminated with the same intensity for the measurement. In this case, soq1 npq4 does not show enhanced quenching compared to npq4, showing that the difference between the growth light and the actinic light is what causes high quenching. To further investigate the role of light intensity and NPQ phenotype, npq4 leaves were measured with three different combinations of growth light and actinic light. When npq4 plants are grown in 25 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \) and illuminated with 2000 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \), the plant has twice the amount of NPQ compared to when plants are grown in 100 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \) and illuminated with 1200 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \). This suggests that either the high actinic light intensity causes more NPQ, or the difference between the growth conditions and the illumination conditions cause a large difference in the amount of NPQ. However, the amount of NPQ for leaves grown in 100 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \) and illuminated with 1200 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \) is similar to the amount of NPQ for leaves grown in and illuminated with 2000 \( \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1} \). This suggests that the difference between the plants’ growth light intensity and illumination intensity is more significant than the actinic light intensity used.

These data suggest that it is possible to observe soq1-like quenching in plants that contain SOQ1 by illuminating with a much higher intensity actinic light compared to the growth light. The data also indicate that growing soq1 in high light is enough to eliminate the extra NPQ in soq1 npq4. However, because the NPQ traces shown in Fig. 4.6 were taken using a PAM fluorometer, any decrease in fluorescence yield, whether or not it is quenching related, will
be interpreted as NPQ. To test whether we could induce soq1 npq4-like quenching in npq4, we measured the fluorescence lifetimes of npq4 for two different growth and illumination conditions:

1). Plants grown in 100 µmol photons × m⁻² × s⁻¹ light and illuminated with 1200 µmol photons × m⁻² × s⁻¹ actinic light to induce quenching (normal growth light intensity and normal illumination light intensity).

2). Plants grown in 25 µmol photons × m⁻² × s⁻¹ light and illuminated with 2000 µmol photons × m⁻² × s⁻¹ actinic light to induce quenching (low growth light intensity and high illumination light intensity).

The average fluorescence lifetimes for npq4 plants exposed to normal and extreme conditions are shown in Fig. 4.7. The plants grown in 100 µmol photons × m⁻² × s⁻¹ light have a shorter average fluorescence lifetime compared to plants grown in 25 µmol photons × m⁻² × s⁻¹ light different average fluorescence lifetimes (1.44 ± 0.05 ns for normal light, 1.56 ± 0.04 ns for low light). While the data shown in Fig. 4.6 suggested that the npq4 plants grown in low light and illuminated with high light would display a shorter average fluorescence lifetime during quenching, they instead display consistently longer average fluorescence lifetimes compared to the npq4 plants grown and illuminated with normal light intensities. In fact, the curves for both plants look nearly identical during the induction of quenching, except with each average fluorescence lifetime for the plants exposed to high light being 100 ps longer than the corresponding measurement for plants exposed to normal light. After the actinic light turns off, both sets of plants experience an increase in quenching typical of the npq4 plants. However, the increase in quenching is greater for the plants exposed to normal light conditions. After this initial drop in average fluorescence lifetime, the average lifetime of both plants increases linearly. However, the plants exposed to extreme light conditions recover more slowly, at a rate of 21 ps per minute compared to the plants grown under normal light conditions, which recover at a rate of 30 ps per minute.

To check how the growth and illumination light intensity affects the amount of NPQ, NPQ was calculated using Eqn. 2.4.2.2. This is shown in Fig. 4.8a. Both exposure conditions lead to the same amount of NPQ and the same induction dynamics. The only major difference between the two plants is in the relaxation. When the actinic light turns off, the plants exposed to normal light conditions experience a greater increase in quenching compared to the plants exposed to extreme light conditions. To confirm the similarity in the amount of quenching, $\phi_{NPQ,RCC}$ was calculated, using Eqn. 2.4.2.4. The curves for both growth and illumination conditions are nearly identical, as well. These curves are shown in Fig. 4.8b.

The similarity between quenching dynamics for npq4 exposed to normal quenching conditions and extreme conditions suggests that the extra quenching seen in Fig. 4.6 is due to signal contamination from photobleaching or chloroplast shielding, and is not actually the same type of quenching seen in soq1 npq4. To test this hypothesis, it is necessary to look at the absolute amount of fluorescence rather than the amplitude-weighted average lifetime. To calculate the average fluorescence intensity for a leaf from one of the growth and illumination conditions, the data from each leaf measurement were aligned, and summed, and divided by the number of leaves measured. The fluorescence decays for npq4 exposed to normal and extreme conditions are shown in Fig. 4.9. Fig. 4.9a. shows the dark-adapted fluorescence
decays. The \textit{npq4} leaves exposed to extreme conditions has a higher maximum intensity compared to \textit{npq4} exposed to normal conditions (389 counts versus 315 counts). After 10 minutes of light, the intensity of the \textit{npq4} leaves exposed to high light is closer to that of \textit{npq4} leaves exposed to normal light (351 counts versus 309 counts). This can be seen in Fig. 4.9b. The 9\% decrease in fluorescence for \textit{npq4} leaves exposed to high light compared to the 2\% decrease in fluorescence for \textit{npq4} leaves exposed to normal light shows that the extra quenching seen in PAM traces of \textit{npq4} grown in extreme conditions is most likely due to photobleaching or other non-quenching processes that decrease the chlorophyll fluorescence yield such as chloroplast shielding, and not due to NPQ. This highlights the utility of the fluorescence lifetime apparatus to specifically detect quenching.

### 4.4.3 Average fluorescence lifetime of \textit{A164}

To further elucidate the role of SOQ1 in quenching, \textit{soq1 npq4} seeds were exposed to a second round of mutations in order to examine what mutations enhance or suppress the \textit{soq1} phenotype. This work was conducted by Alizée Malnoë. Plants that do not show enhanced quenching will be suppressors of the \textit{soq1} phenotype. In the case of \textit{soq1 npq4}, which lacks
Characterizing a new type of NPQ mutant 4.4. Results

Figure 4.8: NPQ of npq4 is shown in a., and $\phi_{NPQ,RCC}$ is shown in b.. The data shown in red corresponds to plants that were grown in 100 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ light and illuminated with 1200 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ actinic light to induce quenching (normal conditions for measuring NPQ), and the data shown in black corresponds to leaves grown in 25 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ light and illuminated with 2000 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ actinic light to induce quenching (extreme conditions for measuring NPQ).

Figure 4.9: The data shown in red corresponds to plants that were grown in 100 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ light and illuminated with 1200 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ actinic light to induce quenching (normal conditions for measuring NPQ), and the data shown in black corresponds to leaves grown in 25 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ light and illuminated with 2000 $\mu$mol photons $\times$ m$^{-2}$ $\times$ s$^{-1}$ actinic light to induce quenching (extreme conditions for measuring NPQ). a. shows the dark-adapted fluorescence decays and b. shows the light adapted fluorescence decays.

the protein SOQ1, a suppressor would likely modify the target of SOQ1 such that it is constitutively active or inactive such that it stays in the state that SOQ1 would normally cause. This is shown in Fig. 4.10.

A false-color image of fluorescence yield from leaves of potential mutants selected during the suppressor screen is shown in Fig. 4.11a. and b. A soq1 npq4 leaf is shown for comparison (circled in pink). There are two leaves that show a reduced $F_m$ compared to soq1 npq4. The leaf circled in blue is A164. The other leaf with reduced $F_m$ is from a plant that likely has the same mutation as A164. The A164 mutant grows well, though more slowly than soq1 npq4.
4.4. Results

Characterizing a new type of NPQ mutant

Figure 4.10: One vision for how SOQ1 may act, and how soq1 and a suppressor of soq1 might behave. In the first column, SOQ1 is imagined to act directly on a light harvesting complex. In the second and third columns, SOQ1 is imagined to act on an intermediate, which then acts on a light harvesting complex. In the second column, the action of SOQ1 is to activate an intermediate, which prevents quenching. In the third column, SOQ1 inactivates an intermediate which would have caused quenching. The first row shows the behavior expected in wild-type. The second row shows the behavior expected in soq1. The third row shows the expected behavior for a suppressor of the soq1-phenotype.

soq1 npq4 is shown on the left and A164 is shown in the right in Fig. 4.11c.

A164 shows a highly quenched phenotype, even when dark-adapted. To investigate the quenching dynamics of A164, the fluorescence lifetimes were measured for A164 over 10
minutes of illumination and relaxation. After 10 minutes of illumination with 1200 $\mu$mol photons $\times$ m$^{-2} \times$ s$^{-1}$ actinic light, the fluorescence lifetime of $A164$ does not change significantly. The dark-adapted average fluorescence lifetime is $0.056 \pm 0.002$ ns and the light-adapted average fluorescence lifetime is $0.052 \pm 0.002$ ns. The fluorescence lifetime decays of $A164$ before and after illumination are shown in Fig. 4.12.

The fluorescence lifetime of $A164$ is also shorter than the strongest quenching measured on any of the mutants. The light-acclimated fluorescence lifetime of $A164$ and $soq1 npq4$ are shown in Fig. 4.13.

The fact that the maximum intensity of a $A164$ leaf is the same as a $soq1 npq4$ leaf indicates that the chlorophyll contents of $A164$ and $soq1 npq4$ are similar, and the reduced $F_m$ is due solely to quenching. The extremely short lifetime of $A164$ and the minimal change in the amount quenching suggests that the measurements may not be taken under conditions of closed reaction centers. To test this, the 5 different steps of a dark-adapted measurement were examined for $A164$ and $soq1 npq4$. The fluorescence decays are shown in Fig. 4.14. The five steps each have a different amount of laser exposure before the measurement, ranging from 0 seconds for the first step to 0.8 seconds for the last step.

For $soq1 npq4$, there is a large difference between the fluorescence decay of step 1, in which the first 200 ms of fluorescence is collected, and steps 2-5, in which subsequent 200 ms periods of fluorescence is collected. This reflects the fact that it takes approximately 200 ms to close the reaction centers, and then the fluorescence yield slowly decreases after this time. For $A164$, there is a very small difference between the fluorescence decays for each step. This suggests that the reaction centers are not closed by the laser during the measurement.
### 4.4. Results

**Characterizing a new type of NPQ mutant**

<table>
<thead>
<tr>
<th>Time (ns)</th>
<th>Average Intensity</th>
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<tr>
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**Figure 4.12:** The fluorescence lifetimes of *A164* in the dark and after 9.5 minutes of actinic light illumination are shown in red and black, respectively. The average fluorescence decay is calculated by dividing the total number of counts by the number of leaves.

Initial EM images of the thylakoids of *A164, soq1 npq4*, and wild-type indicate that while the thylakoid architecture is unchanged between wild-type and *soq1 npq4*, the mutation or mutations responsible for *A164* significantly change the thylakoid architecture. The grana stacks are much larger, and there is little to no stroma lamellae.
Figure 4.13: The fluorescence lifetimes of A164 and soq1 npq4 after 9.5 minutes of actinic light illumination are shown in red and black, respectively. The average fluorescence decay is calculated by dividing the total number of counts for a given mutant by the number of leaves.
Figure 4.14: The fluorescence decays corresponding to different measurements steps are shown for A164 and soq1 npq4.

Figure 4.15: Transmission electron microscopy images are shown in a. and b. The experiment design and image selection was done by Alizée Malnoë.
4.5 Discussion

Measurements of the average fluorescence lifetimes of mutants with and without SOQ1 show that the lack of SOQ1 confers extra quenching, but not a significant amount of extra photobleaching or other processes that reduce the fluorescence yield. Comparing the induction dynamics of soq1 npq4 and npq4, as shown in Fig. 4.2, suggests that SOQ1 specifically affects long-timescale quenching. The lack of SOQ1 reduces the longer timescale of quenching seen in npq4 by a factor of two, from 43 minutes to 22 minutes. It is not known what this quenching corresponds to, since it is longer than the timescales of PsbS-dependent or zeaxanthin-dependent quenching. However, these results suggest that it is regulated by SOQ1.

As shown in Fig. 4.2, the presence of SOQ1 does not impact the dynamics of NPQ relaxation. This suggests that the absence of SOQ1 either introduces a type of quenching that has such a slow rate of turn-off that it is undetectable within the 10 minute relaxation period, or enhances the induction of an existing quenching without changing the turn-off of that quenching.

The fluorescence decay comparisons between wild-type and soq1 and between npq4 and soq1 npq4 shown in Fig. 4.4 indicate that the absence of SOQ1 does not change the excited state dynamics of chlorophyll during quenching. However, these comparisons only look at soq1 and soq1 npq4 during the first few minutes of illumination. Comparing soq1 npq4 during the induction and relaxation of quenching with npq4 during the same times, as shown in Fig. 4.5, shows that while the quenching when SOQ1 is present is reversible within the minutes of darkness, the quenching when SOQ1 is absent is not the same during the induction and relaxation periods. This result would seem to conflict with the idea that SOQ1 does not affect the chlorophyll relaxation dynamics at early illumination times. One way to explain both of these results it is only possible to see that the presence of SOQ1 changes the chlorophyll relaxation dynamics when there is high amounts of quenching. However, another explanation is that the absence of SOQ1 does not change the type of quenching, but that its absence causes the activation of quenching sites that are difficult to deactiate. The difference in the shape of the fluorescence lifetime decay for soq1 npq4 in Fig. 4.5b could be because certain quenching sites are unable to turn off. To further compare the type of quenching when SOQ1 is present to when SOQ1 is absent in the case of high quenching, it would be useful to compare soq1 with L17, an over-expressor of PsbS. This mutant shows high quenching [37], and could therefore be used to compare high qE-type quenching with high soq1-type quenching.

NPQ values calculated from fluorescence yield measurements indicate that enhanced, soq1-type quenching can be seen in plants without SOQ1 if they are illuminated with much higher light than their growth conditions, as shown in Fig. 4.6. However, NPQ measurements derived from fluorescence lifetime measurements show no difference between the plants exposed to normal growth and NPQ conditions and plants exposed to extreme growth and NPQ conditions, as can be seen in Fig. 4.8. Plants grown in low light and induced with high light experience equivalent quenching but higher photobleaching compared to plants grown and induced with normal light (as shown in Fig. 4.9). Because fluorescence yield measurements do not distinguish between photobleaching and quenching, this extra photobleaching is falsely
interpreted as NPQ. There may be a way to override the capacity of SOQ1 and induce $soq1$-type quenching in plants with SOQ1, but illumination with a higher light intensity is not the mechanism to do so. This result also suggests that it would be useful to measure NPQ from fluorescence lifetimes on $soq1$ when illuminated with different light intensities. The higher quenching seen in $soq1$ has only been seen with fluorescence yield measurements that use an actinic light intensity that is much higher than the growth light. While $soq1 npq4$ plants grown and illuminated with $2000 \mu \text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$ do not show enhanced quenching compared to $npq4$ plants grown and illuminated with this light intensity, it could be that the high light causes significantly more photobleaching in $soq1 npq4$ compared to $npq4$. The increase in photobleaching could mask an increase in quenching in fluorescence yield measurements. The relative contributions of quenching and photobleaching in $soq1 npq4$ grown and illuminated in high light could be easily resolved with fluorescence lifetime measurements.

To try to elucidate the target of SOQ1, $soq1 npq4$ suppressors were developed. While the $A164$ mutant shows reduced NPQ, it is in fact due to constitutively high quenching rather than reduced quenching. The fast fluorescence lifetime may be explained by the generation of a new quenching site that is much stronger than normal qE, qT, or qI. Another possibility is that the $A164$ mutant has many more PSII reaction centers, and therefore it takes either a higher light intensity or longer light exposure to close them. Alternatively, it could be that $A164$ has a mutated form of PSII that does not close. Additionally, the plastoquinone pool could be increased such that PSII is no longer acceptor limited. The enlarged grana and smaller stroma lamellae in $A164$ may be a direct effect of the mutation, for example, an increase in lipid production, or it could also be a secondary effect of the mutation. For example, the grana size may be up-regulated to compensate for the high quenching in the $A164$ mutant. This high quenching might be related to PSII, but it could also be that the enlarged grana are accommodating PSI in the LHCII pool, and that the PSI are acting as quenchers. The utility of $A164$ for our understanding of the role of SOQ1 remains to be seen. If the mutation responsible for $A164$ causes high quenching in wild-type, we will learn about the regulation of quenching, but not as much about SOQ1. However, if the mutation only causes high quenching in the absence of SOQ1, it may be used to determine the target of SOQ1.

4.6 Conclusions

We have proven that the lack of SOQ1 confers extra quenching, not extra photobleaching or chloroplast shielding. The absence of SOQ1 has an increasing effect on the amount of quenching during illumination, but no effect on the amount of quenching during relaxation. SOQ1 prevents a quenching process from being turned on in the light, but does not impact the minutes-timescale rate at which quenching turns off. This suggests that either the activation and inactivation of SOQ1 is asymmetric or the activation or inactivation of its target is asymmetric.

Comparisons between the fluorescence decays of dark-adapted plants with and without SOQ1 show that SOQ1 may suppress quenching slightly in the dark. Comparisons between
plants with and without SOQ1 at early illumination times suggest that the presence of SOQ1 does not impact the relaxation dynamics of chlorophyll, but comparisons between the fluorescence decays of plants with and without SOQ1 during the induction and relaxation of quenching suggests that SOQ1 does alter the quenching pathways available at later illumination times. Comparing the dynamics between *soq1 npq4* and *npq4* also suggests that the role of SOQ1 may be specific to long-term quenching.

It is not possible to overwhelm the action of the SOQ1 protein just by using a higher light intensity. Plants that have SOQ1 do not display *soq1*-type quenching when a higher light intensity is used. This suggests that SOQ1 has a high capacity for suppressing quenching.

The *A164* mutant shows constitutive quenching that is not responsive to illumination with actinic light or to different laser exposure time periods. This mutant shows markedly different dynamics than any other plant mutant studied, and, depending on the genes responsible for the quenching, may be a useful system to study the effect of membrane architecture, strong quenchers, or open reaction centers on fluorescence quenching.

### 4.7 Supplemental

#### 4.7.1 Investigating the *D101* mutant

To try to understand the role of SOQ1, we examined a mutant, *D101*, which is a mutant of *soq1 npq4* that shows enhanced *soq1*-type quenching. The average fluorescence lifetimes of *soq1* and *D101* are shown below in Fig. 4.16.

Both *D101* and *soq1 npq4* start out with the same average fluorescence lifetime. When the actinic light turns on, both mutants experience quenching, but within a minute of illumination, *D101* begins to quench more strongly than *soq1 npq4*. After 2-3 minutes of illumination, both the rate of quenching between *D101* and *soq1 npq4* becomes the same, though *D101* has a shorter average fluorescence lifetime than *soq1 npq4* due to its increased rate of induction of quenching during the first few minutes of illumination. When the actinic light turns off, both mutants experience a small increase in quenching, as is common in all the mutants in the *npq4* background. After 30 seconds, both mutants have a linear increase in the average fluorescence lifetime, though the speed of relaxation for *soq1 npq4* is slightly faster than *D101*.

If the mutation in *D101* enhances the *soq1* phenotype, we would expect that the difference between the average fluorescence lifetimes of *D101* and *soq1 npq4* would be similar to the difference between *soq1 npq4* and *npq4*. However, while *soq1* has an increasing effect during the illumination period and no effect on the relaxation, *D101* only affects the earlier illumination times and then decreases the relaxation of quenching. Additionally, *D101* and *soq1 npq4* do not have similar fluorescence decays in the light when the average fluorescence lifetime are similar. This suggests that *D101* is not a model system to study enhanced *soq1*-type quenching.
Figure 4.16: The average fluorescence lifetimes of soq1 npq4 and D101 are shown in black and red, respectively.

4.7.2 Fits to the induction of NPQ in soq1 npq4 and npq4
Figure 4.17: The average fluorescence lifetimes of *npq4* and *soq1 npq4* are shown in top and bottom images, respectively. The data points are shown by circles and the biexponential fit is shown by the blue line.
Chapter 5

Building a STED-FLIM microscope

5.1 Collaborators

The microscope was constructed in the laboratory of Graham Fleming. All STED experiments described were performed in the laboratory of Professor Stefan Hell in the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry. Training on the STED microscopes was done under the supervision of Dr. Brian Rankin, Dr. Volker Westphal, Markus Schuster, and Dr. Haisen Ta. The work described in this chapter is part of the Single-Investigator and Small-Group Research Initiative (SISGR), a collaboration with the groups of Professors Krishna Niyogi, Carlos Bustamante, Dan Fletcher, Eva Nogales, Jan Liphardt, Berend Smit, and Naomi Ginsberg.

5.2 Introduction

During NPQ, the architecture and composition of the thylakoid undergoes multiple changes. High light causes grana membranes to de-stack, increasing the distance between individual grana membranes [114, 115]. Fluctuations in ion concentrations during quenching cause the size of grana stacks to swell [116]. In addition, the size of grana stacks can fluctuate, both in lateral diameter and in number of membranes per stack [117, 118]. It is hypothesized that these changes in membrane architecture modulate the diffusion of proteins in the membrane [119], in turn changing the protein composition of the grana and stroma to balance excitation energy [120], prevent photodamage [121], and facilitate repair [122].

This cycle of PSII photodamage and repair is of particular interest because it has been estimated that without PSII repair, the efficiency of photosynthesis would be less than 5% of its existing efficiency [66]. PSII damage is thought to occur via two main mechanisms: donor-side inhibition, in which the oxygen-evolving complex is damaged either by a low luminal pH [123] or by blue-light-induced destruction of the Mn$_4$-Ca-Cl$_2$ complex [124], and acceptor-side inhibition, in which the D1 protein subunit is damaged by singlet oxygen [125]. There is general consensus that regardless of where photoinhibition initially occurs, the D1 subunit of PSII is the main protein subunit damage [126].
Photoinhibition causes a lowered \( F_m \), which indicates a lowered maximum possible fluorescence yield \([127]\). This lowered fluorescence yield is due to quenching \([128, 129]\). It has been hypothesized that damaged \( \text{PSII} \) complexes act as quenchers to protect the remaining \( \text{PSII} \) from getting further damaged \([7, 130, 129]\). Research on \( \text{PSII} \) repair has suggested that damage and repair occur as follows \([125]\):

1) \( \text{PSII} \) is photoinhibited. This occurs as a first-order reaction with an illumination-dependent rate constant \([131]\).
2) \( \text{PSII} \) is phosphorylated, which causes dissociation of the \( \text{PSII} \) supercomplex. \( \text{PSII} \) subunits migrate to the stroma lamellae \([125]\).
3) \( \text{D1} \) is degraded (the half-time for this process is estimated to be 1 hr \([132]\))
4) \( \text{D1} \) is re-synthesized (variable rate \([133]\)), and migrates from the stroma lamellae to the grana \([125]\).
5) \( \text{PSII} \) is reassembled.

The total amount of time for repair after 4 hours of illumination with high light (2000 µmol photons·m\(^{-2}\)·s\(^{-1}\)) is approximately 4 hours \([40]\).

The damage and repair cycle of \( \text{PSII} \) is hypothesized to involve lateral migration of proteins between the grana and stroma lamellae. To confirm this process, as well as to determine the precise timescales of each of the steps, it is highly desirable to have a technique capable of visualizing \( \text{PSII} \) supercomplexes and subunits throughout the repair process. However, developing a microscope with this capability is challenging for the following reasons:

1) The size of a photosystem II complex is roughly 20 nm by 30 nm \([134]\). This is over an order of magnitude smaller than what can be visualized by confocal fluorescence microscopy \([135]\).
2) Due to tight stacking of the thylakoid membrane, bulky fluorophore tags such as GFP can disrupt the grana stacking and hinder protein diffusion \([30]\).
3) The broad absorption of pigments in the thylakoid membrane limit the potential spectral range of fluorophore tags.

To be able to resolve \( \text{PSII} \) in the thylakoid membrane and observe \textit{in vivo} dynamics, it is necessary to use super-resolution optical microscopy. Because of the complication in tagging \( \text{PSII} \), we decided to use the endogenous fluorescence of chlorophyll as the fluorophore.

### 5.3 Theory of STED Microscopy

The ability of a microscope objective to resolve closely spaced objects is limited by diffraction \([136]\). A point source of light, when imaged, will appear as a bright circle, surrounded by rings of light. This pattern, the point spread function (PSF) of a point source, is referred to as an Airy diffraction image, and is due to the fact that a lens does not collect the entire wavefront of emitting light, and therefore does not have enough information to accurately recreate an image of the of the point source. The distance that is commonly used as the minimum distance at which two point sources can be resolved is equal to the distance between the center of an Airy disk and the first dark ring, \( r_{\text{Airy}} \):
5.3. Theory of STED Microscopy

Building a STED-FLIM microscope

\[ r_{\text{Airy}} = 0.61 \frac{\lambda}{NA_{\text{obj}}} \]  

(5.3.0.1)

\( \lambda \) is the wavelength of the illumination light in a vacuum, and \( NA_{\text{obj}} \) is the numerical apertures of the objective [135]. For 700 nm red light and the highest numerical aperture generally used, of 1.4, \( r_{\text{Airy}} \) equals 305 nm.

For over one hundred years, this minimum distance, also referred to as the Rayleigh criterion, was thought to be the limit of optical imaging. However, in the late twentieth century, different strategies were developed to try to get higher resolution. One strategy is to image objects in the near-field, on the order of tens of nanometers from the excitation source. The Rayleigh criterion assumes far field propagation of light. Near-field scanning optical microscopy (NSOM) uses evanescent waves to excite fluorophores in a sub-diffraction volume [137]. However, because evanescent waves decay within 100 nm, the utility of NSOM is limited to imaging of surfaces. In the late 1990s, two different concepts for achieving super-resolution in the far-field were developed. One of these concepts is localization microscopy. Localization microscopy involves exciting a diffraction-limited volume, and collecting enough photons from each fluorophore to determine the center of each fluorophore’s PSF with higher accuracy. The position of each fluorophore can then be localized to the center of the PSF. The ability to localize a photon can be approximated by \( \frac{s}{\sqrt{N}} \), where \( s \) is the standard deviation of the PSF and \( N \) is the number of photons [138, 139]. Localization microscopy techniques include stochastic optical reconstruction microscopy (STORM) [140] and photoactivated localization microscopy (PALM) [141]. Localization microscopy techniques often require long imaging times to accumulate sufficient individual excitation events of each fluorophore, and the use of particular fluorophores that can be re-excited many times.

In 1994, Stefan Hell and Jan Wichmann proposed a microscopy technique that would break the diffraction limit by reducing the PSF of fluorophores [142]. Their idea was to excite a diffraction-limited volume in a sample, but then, to de-excite the sample through stimulated emission around the perimeter of the excited spot such that only a smaller, sub-diffraction size spot could fluoresce [143]. This technique was named stimulated emission depletion microscopy (STED). The transitions induced by interactions with the excitation beam and stimulated emission beam are shown schematically in Fig. 5.1a. The wavelengths for the absorption, fluorescence and stimulated emission for a sample fluorophore are shown in Fig. 5.1b.

To efficiently deplete the periphery of the excited sample, it is desirable to use a doughnut-shaped beam. Overlaying the depletion beam with a Gaussian-shaped excitation beam means that the only area that is allowed to fluoresce is the “doughnut hole.” This is shown schematically in Fig. 5.2.

A cross section of the two laser beams is shown below in Fig. 5.3.

The resolution of STED microscope is defined, in part, by the intensity of the laser beam. The derivation of the PSF of a STED microscope is shown below, based on references [144, 145].
5.3. Theory of STED Microscopy

Figure 5.1: The energy level diagram for a sample fluorophore is shown in a. Absorption occurs from the ground vibrational level of the ground electronic state ($S_0$) to a vibrationally excited level in an electronically excited state ($S_1$). Following excitation, the fluorophore undergoes vibrational relaxation to the ground vibrational level of $S_1$. After this time, the depletion beam is exposed to the periphery of the excited sample. It causes relaxation of the fluorophores to a vibrationally excited level of $S_0$. For the portion of the sample that is not exposed to the depletion beam, fluorescence will occur, allowing the fluorophores to relax to the ground vibrational level of $S_0$. The energies of the relevant transitions are shown with respect to the absorption and emission spectra of a fluorophore in b.

Figure 5.2: A schematic of the lateral spatial profiles of the excitation (blue) and depletion (orange) beams are shown. The center of the excitation beam that is not exposed to the stimulated emission beam fluoresces.

The PSF of the “doughnut hole” can be described as a function of the excitation area and the depletion area:

$$PSF = (\text{Area excited}) \cdot (\text{Area depleted})$$  \hspace{1cm} (5.3.0.2)

The excited area is a Gaussian:
5.3. Theory of STED Microscopy

Building a STED-FLIM microscope

Figure 5.3: The intensity profiles of the excitation (blue) and depletion (orange) beams. The intensity profiles are normalized to the same maximum intensity for clarity, though in the experiment, the intensity of the depletion beam is many orders of magnitude greater than the excitation beam.

\[
\text{Area excited} = e^{-4 \ln 2 \cdot \frac{x^2}{d_C^2}}
\]  
(5.3.0.3)

where the diameter of the excitation beam, \(d_C\), is defined by the diffraction limit:

\[
d_C = \frac{1.22 \cdot \lambda}{2 \cdot NA}
\]  
(5.3.0.4)

The area that is depleted can be described as a Gaussian with decreasing intensity at the center of the beam:

\[
\text{Area depleted} = e^{-I_{\text{STED}}(x) \cdot \sigma_{\text{STED}} \cdot \tau}
\]  
(5.3.0.5)

In the above equation, \(\sigma_{\text{STED}}\) is the absorption cross-section of the depletion beam, \(\tau\) is the amount of time over which the sample is exposed to the depletion beam, \(I_{\text{STED}}\) is the spatial intensity of the depletion beam, which can be approximated as a parabola around the center of the Gaussian beam:

\[
I_{\text{STED}}(x) = 4 \cdot a^2 \cdot x^2 \cdot I_{\text{STED}}
\]  
(5.3.0.6)

In the above equation, \(a\) is the steepness of the parabola, \(I_{\text{STED}}\) is the maximum intensity of the depletion beam, \(x\) is the spatial coordinate, and 4 is a scaling factor.

The area that is depleted can then be expressed as follows:
Area depleted \( = e^{-4a^2x^2I_{STED}\sigma_{STED}\tau} \) (5.3.0.7)

The area that is depleted also depends on the relaxation dynamics of the excited state, \( S_1 \). The time evolution of the population of \( S_1 \), \( N_1 \), can be described as follows:

\[
\frac{dN_1}{dt} = -(k_{fl} + k_{STED}) \cdot N_1(t) \quad (5.3.0.8)
\]

In the above equation, \( k_{fl} \) is the rate of fluorescence, and \( k_{STED} \) is the rate of depletion by the depletion beam. Immediately after excitation, \( S_1 \) is populated:

\[
N_1(0) = 1 \quad (5.3.0.9)
\]

As time goes on, the population of \( S_1 \) will relax, eventually repopulating \( S_0 \):

\[
N_1(t \to \infty) = 0 \quad (5.3.0.10)
\]

Solving \( \frac{dN_1}{dt} \) for \( N_1 \) gives the following:

\[
N_1(t) = e^{-(k_{fl} + k_{STED})t} \quad (5.3.0.11)
\]

The rate of depletion, \( k_{STED} \) is also a function of the intensity of the depletion beam, \( I_{STED} \), and the depletion cross-section, \( \sigma_{STED} \). We can therefore re-write Eqn. 5.3.0.11 as follows:

\[
N_1(t) = e^{-(k_{fl} + I_{STED}\sigma_{STED})t} \quad (5.3.0.12)
\]

After the depletion beam has been on the sample for a duration \( \tau \), the population of \( S_1 \) is as follows:

\[
N_1(\tau) = e^{-(k_{fl} + I_{STED}\sigma_{STED})\tau} \quad (5.3.0.13)
\]

Comparing the population of \( S_1 \) with and without the depletion beam gives us a suppression factor, \( \eta \), which is dependent on the intensity of the depletion beam:

\[
\eta(I_{STED}) = \frac{N_1(I_{STED})}{N_1(I_{STED} = 0)} \quad (5.3.0.14)
\]

Plugging in Eqn. 5.3.0.13, we get the following:

\[
\eta(I_{STED}) = \frac{e^{-(k_{fl} + I_{STED}\sigma_{STED})\tau}}{e^{-k_{fl}\tau}} \quad (5.3.0.15)
\]

This simplifies:

\[
\eta(I_{STED}) = e^{-I_{STED}\sigma_{STED}\tau} \quad (5.3.0.16)
\]

The intensity of the depletion beam that de-excites half of the fluorophores, \( I_S \) can therefore be defined as follows:
5.3. Theory of STED Microscopy

Building a STED-FLIM microscope

\[
\frac{1}{2} = e^{-I_S \sigma_{STED} \tau} \quad (5.3.0.17)
\]

Solving for \(\sigma_{STED}\), we get:

\[
\sigma_{STED} = \frac{\ln 2}{I_S \cdot \tau} \quad (5.3.0.18)
\]

Plugging this into Eqn. 5.3.0.7, we get

\[
\text{Area depleted} = e^{-4 \ln 2 \cdot a^2 \cdot x^2 \cdot \frac{I_{STED}}{I_S}} \quad (5.3.0.19)
\]

Combining Eqn. 5.3.0.5 and Eqn. 5.3.0.19 into Eqn. 5.3.0.2, we get:

\[
PSF = e^{-4 \ln 2 \cdot x^2 \left(\frac{1}{\sigma^2} + a^2 \cdot \frac{I_{STED}}{I_S}\right)} \quad (5.3.0.20)
\]

This PSF of the remaining excited region describes a Gaussian with a full-width at half-maximum (FWHM), \(d\), where:

\[
d = \frac{d_C}{\sqrt{1 + \left(d_C^2 \cdot a^2 \cdot \frac{I_{STED}}{I_S}\right)}} \quad (5.3.0.21)
\]

This equation says that the FWHM of the excitation spot size depends on the excitation spot size, and on the ratio, \(\frac{I_{STED}}{I_S}\). The higher the intensity of the depletion beam with respect to the intensity needed to deplete half the fluorophores, the smaller the FWHM of the excited spot will be, and the higher the microscope resolution will be.

Multiple techniques have been used to generate a doughnut shaped laser beam. The first experiments were performed by splitting the depletion beam in two, and displacing one depletion beam on the top and bottom of the excitation spot size [143, 146]. Later, a doughnut was created by phase shifting different portions of the depletion beam such that the two vertical halves and two horizontal halves of the beam each had a \(\pi\) phase difference [147]. This causes destructive interference at the center of the STED beam. Later, a spatial light modulator (SLM) was used to generate a circularly polarized doughnut-shaped beam [148]. However, due to pixelation effects, the use of the SLM was discarded in favor of a vortex phase mask [149]. The vortex phase mask that we employed is produced by RPC Photonics, and consists of a glass plate with a polymer deposited such that any two points 180° apart experience a \(\pi\) phase shift. This creates a phase singularity, otherwise thought of as destructive interference at the center of the beam [150].

The action of the phase mask can be simulated by applying the vortex phase to a Gaussian beam, shown below.

Immediately after the vortex phase is applied to the Gaussian beam, it does not look different. For the destructive interference to take place, the beam must propagate for meters. This is shown below.

The distance required to propagate the beam can be reduced by the use of a lens, which applies an additional phase to the beam that increases the interference. Images of the laser beam before and after focusing with a lens are shown below.
Figure 5.4: A simulation of a Gaussian beam is shown in \( a \). A simulation of a vortex phase mask is shown in \( b \). The phase from 0 to \( 2\pi \) is represented by the shade, ranging from white to black.

Figure 5.5: A simulation of a Gaussian beam after a vortex phase is applied is shown for different propagation distances. In \( a \), the beam has been propagated for 0.1 meters. \( b \) shows the beam after 10 meters of propagation, and \( c \) shows the beam after 1000 meters of propagation.
5.3. Theory of STED Microscopy

Building a STED-FLIM microscope

Figure 5.6: Images taken with a beam profiling camera for the laser beam before and after passing through the vortex phase mask and lens are shown in a. and b.
5.4 Design

STED microscopy is best used when there is a low concentration of fluorophores so that each tagged protein can be easily distinguished. However, the grana stack is composed of approximately 80% protein [4]. The most abundant complex in the thylakoid, the light harvesting complex II (LHCII) [5], binds chlorophyll, and would therefore most likely overwhelm any chlorophyll fluorescence from Photosystem II (PSII). To be able to distinguish PSII from LHCII, it is necessary to use STED microscopy in combination with fluorescence lifetime imaging (FLIM). STED-FLIM microscopy was developed by the group of Paul French [151]. In STED-FLIM microscopy, the contrast in an image is based on the fluorescence lifetime of each pixel rather than the fluorescence intensity of each pixel. Because PSII has distinct fluorescence lifetime components from other chlorophyll-binding proteins [70], by measuring the fluorescence lifetime of each pixel, we will be able to identify which protein or sets of proteins are fluorescing.

To image PSII damage and repair with STED-FLIM, isolated thylakoids from dark-adapted plants would be first be imaged. Then, the thylakoids would be exposed to a white light source at a high enough intensity to cause significant photodamage (2000 μmol photons·m⁻²·s⁻¹). Then, the thylakoids would be imaged again. Based on the evidence for damaged PSII complexes migrating to the stroma lamellae, we would expect to see less fluorescence in the grana and more in the stroma lamellae. Additionally, because damaged PSII complexes are expected to act as quenchers, these complexes would likely have a shorter fluorescence lifetime. After hours of repair, we would expect images of the thylakoid membrane to resemble that of the thylakoids from dark-adapted plants. Cartoons of the expected results are shown in Fig. 5.7.

Most STED microscopes use pulsed laser sources for the excitation and depletion beams. The pulsing allows for temporal separation between the excitation and depletion beams, and for a low time-averaged intensity of both beams on the sample [152]. Two different laser sources are generally used because the wavelengths of the maxima of the excitation and depletion beam are generally separated by 100 to 200 nm, though this varies based on the Stokes shift of the fluorophore used (see http://nanobiophotonics.mpiibpc.mpg.de/old/dyes/). The excitation source is generally a pulsed nanosecond laser, and the depletion source is frequently a mode-locked Ti:Sapphire laser. Continuous wave (CW) depletion sources can be used but are less efficient at depletion due to their lower intensity [152].

To perform STED-FLIM microscopy, it is necessary to use an excitation pulse that is shorter than the instrument response function (IRF) of the detector. Otherwise, the fluorescence cannot be significantly localized in time. Because the IRF of our detectors (Hamamatsu R3809-U) is <30 ps, it makes sense to use the Ti:Sapphire laser to generate both the excitation and depletion beams. Using the Ti:Sapphire laser for both beam is challenging because the requirements of the excitation and depletion beams differ significantly:

1) Pulse duration: the pulse duration of the excitation beam should be <30 ps. However, the pulse duration of the depletion beam needs to be stretched to > 50 ps, and is generally stretched to >200 ps [148]. This stretching reduces the amount of re-excitation by the depletion beam [143] and prevents photodamage due to nonlinear absorption [153].
2) Pulse spectrum: the excitation beam needs to be absorbed by Photosystem II, which has a broad absorption in the chlorophyll Soret band (400-450 nm) and in the Qy band, which extends from 650 nm to 690 nm, and peaks at 671.8 and 678.6 nm [154]. Because excitation of the Soret band results in fast internal conversion to the Qy band, we decided to excite the Qy band directly to ensure more efficient depletion. The depletion beam needs to be at the red edge of the fluorescence in order to cause stimulated emission from the vibrational ground state of $S_1$ to a vibrationally excited state of $S_0$. Based on the fluorescence spectrum of PSII [154] and of the grana membrane [155], the depletion wavelength should be between 740 and 790 nm.

The absorption and fluorescence spectra of the center of the grana membrane of spinach, which contains mostly LHCII and PSII is shown in Fig. 5.8. The excitation region is defined at wavelengths below 670 nm because above 670 nm, there is significant fluorescence. The detection region is defined as the fluorescence extending from 670 nm to 740 nm. Below 670 nm, there is significant absorption, and above 740 nm, it is desirable to cause depletion.

To create two beams with the appropriate characteristics for the excitation and depletion beams, the Ti:Sapphire output (Coherent Mira) is set to the wavelength for the depletion beam, but has a pulse duration that is better suited for the excitation beam due to the ultrafast pulses that are shorter than the 30 ps required. The Ti:Sapphire output is split into two parts, one part to form the excitation beam, and one part to form the depletion beam. The schematic of the optics leading up to the microscope is shown in Fig. 5.9.

The excitation beam is focused into a supercontinuum generation fiber (NKT Photonics...
Figure 5.8: The absorption (left) and fluorescence (right) spectra of the grana core, altered from [155]. The excitation region is shown in yellow. The detection region is shown in red. The depletion region is shown in blue.

Figure 5.9: Schematic of the optical set-up.

The absorption (left) and fluorescence (right) spectra of the grana core, altered from [155]. The excitation region is shown in yellow. The detection region is shown in red. The depletion region is shown in blue.

The spectrum following pumping with 780 nm is shown in Fig. 5.10. Though the spectrum is also broadened temporally, the 11 cm of fiber only causes an approximately 2 ps delay between the blue and red ends of the spectrum [156], which is still an order of magnitude lower than the IRF of the detector.
Running the light through the supercontinuum generation fiber gives flexibility in the excitation wavelength and also cleans up the beam mode. After spectral broadening, the excitation wavelength is selected with a dichroic filter, delayed using a delay stage, and telescoped to overfill the microscope objective. A photograph of the supercontinuum fiber and filter are shown in Fig. 5.11

The remaining 95% of the Ti:Sapphire output is used to generate the depletion beam. To stretch the pulse, 700 m of single-mode fiber is used (Thorlabs FS-LS-4616). However, because the high power of the depletion beam can cause spectral broadening in the fiber, the pulse is pre-stretched with 0.5 m of highly dispersive glass (Schott SF6). A photograph of this pulse stretching set-up is shown in Fig. 5.12.
Figure 5.12: The depletion beam passes through 0.5 m of highly dispersive glass (SF6) before being coupled into 700 m of fiber to broaden it. The fiber wheel is suspended above the laser table and is not shown in this picture.

After the fiber, the beam passes through the vortex phase mask (RPC Photonics VPP-1a). The vortex phase mask consists of a glass plate with a 5 by 5 grid of vortex phase masks for different wavelengths. A photograph of the vortex phase mask is shown in Fig. 5.13.

Figure 5.13: After spectral broadening, the depletion beam passes through the vortex phase mask.
After telescoping the beam to the appropriate size to overfill the microscope objective, both beams are periscoped into the microscope, which is a modified Nikon Ti-U Eclipse. The microscope has been altered to accommodate a second input beam. It is shown in Fig. 5.14. Once in the microscope, the excitation beam bounces off of a 670 nm long pass dichroic mirror (Chroma 670dclp). The depletion beam bounces off of one of three long pass filters for 700 nm, 720 nm, or 740 nm (Chroma t700dcspxxr-uv, t720dcspxxr-uv, t740dcspxxr-uv) depending on the depletion wavelength used. Both beams pass through a $\lambda/4$ waveplate to circularly polarize the depletion beam to make an even doughnut [147]. The beams are then focused onto the sample using a 100X 1.4 NA oil immersion objective (Nikon CFI Plan Apo). The position of the sample is scanned using a three-axis piezo stage (Physik Instrumente P-561K005 with E-710 controller). The fluorescence is collected back through the objective, and is then transmitted through both dichroics and an emission filter (Chroma Et605lp). The fluorescence can then be directed to a custom fiber coupler (FLG-SMA/2, CF-3), and sent to the detector (Hamamatsu R3809-U). It can also be directed to a CCD camera (Andor Clara).

Figure 5.14: A schematic of the microscope body.
5.5 Results

Before testing of the microscope was completed, images were collected on a STED microscope in Stefan Hell’s group at the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry. This microscope used a Ti:Sapphire laser set to 785 nm (Coherent Mira). Pulses were stretched with glass rods to 200 ps. A separate 635 nm pulsed laser diode (Picoquant) at 2 $\mu$W was used for excitation. To assess the effectiveness of STED microscopy using chlorophyll as a fluorophore, images were compared with and without the depletion beam. The images without the depletion beam are confocal images, and the images with the depletion beam are STED images.

5.5.1 Pulsed STED Imaging

To compare the effectiveness of STED with chlorophyll to STED on a STED-responsive dye, the confocal and STED images were first assessed using sub-diffraction (20 nm) diameter fluorescent beads coated in Atto 647. The use of the depletion beam significantly reduces the FWHM of the beads.

![Confocal Image](image1.png)  ![STED Image](image2.png)

**Figure 5.15:** Images of 20 nm diameter fluorescent beads taken in the STED microscope with and without the depletion beam are shown in the left and right frames, respectively. The average FWHM of the beads when the depletion beam is used (50 nm) is under a third of the FWHM of the beads when the depletion beam is not used (160 nm).

Chloroplasts were isolated and de-enveloped following the protocol described in [115]. With a depletion beam power of 200 mW, the image of chloroplasts is much blurrier and dimmer than in the confocal image. This is shown in Fig. 5.16. The maximum photon count
in a pixel for the confocal image is 1713 and the maximum photon count in a pixel for the STED image is 470.

Figure 5.16: Images of de-enveloped chloroplasts are shown with and without use of the depletion beam in the right and left images, respectively. The image frame size is 7.5 µm by 7.5 µm. The excitation power is 2 µW and the depletion power is 200 mW.

To assess whether the dimness in the STED image is due to depletion or photobleaching, the images were taken twice more with and without the depletion beams. The third confocal and STED images are shown in Fig. 5.17. Both images show less fluorescence with increasing exposure to the depletion beam. After an additional scan with and without the depletion beam, the maximum photon count in a pixel is 84 for a confocal image and 51 for a STED image.

Figure 5.17: Images of de-enveloped chloroplasts are shown after 4 scans (left) and 5 scans (right). The image frame size is 7.5 µm by 7.5 µm. The excitation power is 2 µW and the depletion power is 200 mW.

The decreasing fluorescence suggests that photobleaching of the sample is occurring. This would occur if the sample is absorbing the depletion beam. To check whether the chloroplast is absorbing the depletion beam, chloroplasts were imaged with only the depletion beam. If there is no absorption of the depletion beam, the sample should appear dark. The images are shown in Fig. 5.18. The maximum counts in a pixel is 94 counts after a single scan of
the depletion beam, and 41 counts after another scan of the depletion beam, indicating that multi photon absorption of the depletion beam causes the photobleaching seen in Fig. 5.16 and Fig. 5.17.

![Image](image.png)

**Figure 5.18:** Images of de-enveloped chloroplasts are shown after 1 (left) and 2 (right) scans with the depletion beam. The chloroplasts are not excited with the excitation beam.

### 5.5.2 CW STED Imaging

To decrease multi-photon absorption, the depletion beam was changed to CW mode. This reduces the peak intensity of the depletion beam. Generally, achieving comparable resolution to pulsed STED requires an approximate 4-fold increase in power. However, to see if comparable resolution to confocal microscopy could be achieved with the use of the depletion beam, the average power was kept constant at 200 mW. The comparative resolution of fluorescent beads with and without the use of a CW depletion beam are shown in Fig. 5.19. The resolution of CW STED is twice as large as with pulsed STED when equivalent average powers are used. Next, BBY particles were isolated following the protocol described in [FIND NAME OF PROTOCOL]. The confocal and CW-STED images are shown in Fig. 5.20. The CW-STED image is still blurrier than the confocal image, as well as dimmer (125 counts versus 353 counts in the pixel with the highest counts). This indicates that increasing the power of the depletion beam would only photobleach the sample further.

### 5.5.3 Time-gated STED Imaging

Though multi-photon absorption and photobleaching occurs for both pulsed and CW depletion beams, we hypothesized that we could eliminate fluorescence resulting from multi-photon absorption by time-gating the fluorescence, only allowing photons emitted over a particular time period to be binned for an image. Time-gating was first used to suppress background fluorescence. Using STED-FLIM, Auksorius and co-workers noted that the fluorescent photons with the earliest arrival times do not contain any super-resolution information [151]. This is because the likelihood of the depletion beam causing stimulated emission depends on the amount of exposure time, as shown in Eqn. 5.3.0.17. By only detecting fluo-
5.5. Results

Building a STED-FLIM microscope

Figure 5.19: Images of 20 nm diameter fluorescent beads taken in the STED microscope with and without the depletion beam are shown in the left and right frames, respectively. The average FWHM of the beads when the depletion beam is used (100 nm) is approximately two thirds of the FWHM of the beads when the depletion beam is not used (160 nm).

Figure 5.20: Images of BBY membranes are shown after 1 (left) and 2 (right) scans with the depletion beam. The chloroplasts are not excited with the excitation beam.

cence emitted over a particular range of times, it is possible to target fluorescence occurring in the sub-diffraction “doughnut hole” [157]. A series of time-gated CW STED images are shown in Fig. 5.21. Three of the time steps, ranging from 656 ps to 1.15 ns show comparable resolution to the confocal image of BBY membrane in Fig. 5.20, but none of the steps show increased resolution. This suggests that part of the problem in imaging with chlorophyll is that chlorophyll fluoresces before it can be depleted. This is likely because, unlike in a system with a small number of fluorophore tags, there is significant energy transfer in this system. The thylakoid membrane is designed to quench energy by the reaction centers. This reduces the fluorescence lifetime of the chlorophyll such that most of the chlorophyll likely fluoresces before it is exposed to the depletion beam for long enough to cause significant stimulated emission.
5.6 Discussion

While we had hoped to use STED-FLIM microscopy to image PSII damage and repair, ultimately, we were unable to perform achieve super-resolution using chlorophyll as a fluorophore. Because the resolution was equal to or poorer than confocal microscopy, we would be unable to resolve differences between the protein composition of the grana and stroma lamellae. This low resolution would prevent the localization of intact and damaged PSII in the thylakoid membrane, as imagined in Fig. 5.7.

The reason for the poor resolution with chlorophyll is likely due to undesirable interactions between chlorophyll and the excitation and depletion beams. Illumination with the excitation and depletion beams can cause many other processes besides the ones shown in Fig. 5.1a [158, 159]. These alternative pathways are shown in Fig. 5.22. For instance, the fluorophore can absorb the probe beam following excitation, as shown by the transition labeled 1. These higher energetic states could fluoresce, undergo internal conversion, or result in photobleaching directly [158]. The fluorophore could also undergo multi-photon absorption of the depletion beam before relaxing via fluorescence or internal conversion. This process is labeled 2 in Fig. 5.22. Additionally, the excited fluorophore could undergo intersystem crossing to a triplet state, as shown by the transition labeled 3. Once in a triplet state, it can react with ground state oxygen, $^3\text{O}_2$, to form singlet oxygen, $^1\text{O}_2$. The reactivity of singlet oxygen can result in photobleaching. In order for STED to be efficient, stimulated emission from
$S_1$ must be the dominant interaction between the fluorophore and the depletion beam. An additional requirement is that the fluorophore must have a long enough fluorescence lifetime to allow exposure to the depletion beam to have a significant depletion effect.

Figure 5.22: A subset of possible interactions with the excitation and depletion beams are shown in this Jablonski diagram. The processes labeled 1, 2, and 3 are undesirable interactions between chlorophyll and the excitation and depletion beams.

In the case of chlorophyll, stimulated emission does not seem to be the dominant interaction with the depletion beam. The fluorescence due to exposure to the depletion beam suggests that there is a significant amount of multi-photon absorption, and the high level of photobleaching suggests oxidative damage, either due to a high energy singlet state or from a triplet state. Shifting the wavelength of the depletion beam would likely not have an effect. Two photon absorption of LHCII has been observed with excitation wavelengths as low as 650 nm [160]. The two photon excitation of a leaf shows an increase from 780 to 910 nm [161]. Essentially, any wavelength that shows promise for depletion is also a strong candidate for two-photon absorption into the chlorophyll Soret band. In addition, the fluorescence resulting from two photon excitation overlaps with that from the Qy band [162]. This means that it would be impossible to separate the fluorescence resulting from one and two photon excitations spectrally. Because STED microscopy relies on the ability to deplete chlorophyll
and there is not a wavelength than can easily deplete the excited state of chlorophyll without simultaneously populating excited states, it does not have much promise as a technique to study chlorophyll.

Despite the difficulty developing STED imaging for chlorophyll, there are other ways in which damage and repair in photosynthetic membranes can be studied. Using the fluorescence lifetime apparatus in conjunction with various A. thaliana mutants and treatments, it will be possible to measure the timescales of the different steps in PSII repair. Additionally, by examining the fluorescence lifetimes on leaves that overly accumulate different damaged PSII intermediates, it will be possible to understand what protein complex acts as a quencher. Recently, it was discovered that two kinases are responsible for phosphorylating PSII: STN7 and STN8 [163]. A stn7 stn8 double mutant was developed which is unable to phosphorylate PSII [40]. This means that if PSII becomes damaged, the PSII supercomplex will not be able to dissociate and the plant will therefore accumulate damaged PSII supercomplexes. There are other mutants that are unable to degrade damaged D1 due to mutations to the proteases [164, 165, 166]. These plants will be able to accumulate damaged D1 that cannot be degraded. Additionally, leaves can be vacuum infiltrated with lincomycin, a chemical inhibitor that stops the synthesis of the D1 protein [167]. In leaves treated with lincomycin, damaged PSII subunits will not be able to be resynthesized. We have recently developed an apparatus that is capable of measuring the fluorescence lifetimes of intact leaves at multiple time points during the induction or relaxation of NPQ explained in Chapter 2. We will be able to compare the fluorescence lifetime of photoinhibited wild-type leaves, leaves that are unable to dissociate PSII supercomplexes, leaves that are unable to degrade D1, and leaves that are unable to resynthesize D1. By seeing which of these systems has the strongest quenching, we will be able to determine what form of photoinhibited PSII serves a photoprotective role.

5.7 Conclusions

STED microscopy requires the use of a high intensity depletion beam. Whether pulsed or CW, this beam causes higher multi-photon absorption compared to stimulated emission, and therefore cannot be used to achieve super-resolution images with chlorophyll. Time-gating the fluorescence is a powerful technique to minimize the amount of early fluorescence before there has been significant exposure to the depletion beam. However, it does not solve the problem of fluorescence from multi-photon absorption. Rather, bulk fluorescence lifetime measurements offer a way to elucidate similar information about PSII damage and repair, since spatial resolution is not necessary to understand the timescales of PSII repair, and which damaged PSII intermediates serve quenching roles.
Chapter 6

Conclusions

While NPQ has been studied for over 45 years, there is still much unknown about the feedback loops that control the amount of quenching, about the proteins and enzymes involved in quenching and how they are involved, and about the photophysical mechanism of quenching. There are multiple reasons for the difficulty in trying to understand NPQ. One reason is that the processes associated with NPQ are physically buried in the cell. Many of these processes occur in the lumen, the space within a single granum, such as the lowering of the pH, flow of ions, and the activation of violaxanthin de-epoxidase enzyme (VDE). If the granum was split open for easier investigation, these processes would be perturbed to the point where they would no longer be reflective of the processes in vivo. Additionally, many of the proteins involved in photosynthesis are transmembrane proteins, which are difficult to isolate and crystallize for structural analysis [168]. Another challenge in studying NPQ is the fact that many components play multiple roles. For instance, carotenoids play two seemingly opposite roles as light collectors and photoprotectors [169]. Therefore, carotenoid mutants may display altered dynamics due to a difference in light collection rather than just photoprotection. An additional challenge in studying NPQ is the need for both spatial and temporal resolution on many length and time scales. NPQ is thought to involve rearrangements of proteins on the scales of 10s to 100s of nanometers [170], as well as larger changes in the thylakoid architecture [121]. Energy transfer between chlorophylls within a single photosystem II (PSII) supercomplex occurs on a timescale of hundreds of picoseconds [10], while NPQ turns on and off in seconds to hours, [35, 41].

To study the changes in NPQ, it is necessary to understand how the arrangement of proteins changes during quenching and how energy transfer changes during quenching. This thesis describes the development of two different time-resolved spectroscopic techniques to study non-photochemical quenching (NPQ) in plants: the goal of one technique is to probe changes in the location of different photosynthetic pigment-protein complexes in isolated thylakoid membranes through STED microscopy, and the other technique probes changes in energy transfer by measuring fluorescence lifetimes on leaves. While the STED microscopy techniques discussed in Chapter 5 were not able to create super-resolution images of chloroplasts, there is still a need for high spatial resolution in NPQ studies. While there has been one technique, ground-state depletion with individual molecule return microscopy (GSDIM),
that has been able to reach approximately 40 nm resolution of chlorophyll in chloroplasts [171], there is no way to determine which protein in which the chlorophyll is fluorescing. Without any protein specificity, it is impossible to determine how the arrangement of proteins changes during quenching. This example shows that super-resolution imaging of chlorophyll fluorescence does not give enough information to further understand NPQ.

To understand how energy transfer within the thylakoid membrane changes during quenching, we developed a new technique to measure fluorescence lifetimes on leaves. This technique, described in Chapter 2, is able to correlate the picoseconds to nanoseconds timescale of energy transfer within the thylakoid membrane with the longer timescale of quenching turning on and off in seconds to tens of minutes. The utility of this technique can be seen in its application to gain new information on well-established mutants, as discussed in Chapter 3, and to characterize new mutants, as discussed in Chapter 4. Because the fluorescence lifetime technique is easily used on any leaf, it can, in the future, be used to investigate other NPQ mutants, as well as non-model plants. In addition, leaves could be vacuum-infiltrated with chemical inhibitors before or during illumination. Treatments such as nigericin, which inhibits the formation of a pH gradient across the thylakoid membrane, diaminodurene, which enhances a pH gradient, and valinomycin, which prevents $\Delta_\psi$ formation, can be used to manipulate the amount of NPQ and the speed with which NPQ turns on and off [90].

No single technique is likely to be the magic bullet to solve NPQ. Imaging techniques only inform on the location of proteins, but cannot directly probe the energy transfer dynamics. Fluorescence lifetime measurements can investigate the energy transfer dynamics of chlorophyll, but are yet unable to give information that is sufficiently spatially-resolved to understand how the protein arrangement and membrane architecture change. Additionally, the fluorescence lifetime measurements are unable to give information about the photophysical mechanism of quenching, whether it is through chlorophyll-chlorophyll interactions [25, 172, 173], chlorophyll-carotenoid interactions [174, 175, 28, 29, 176, 61], or a combination of the two interactions. For these reasons, a complete understanding of NPQ will likely come from combining different experimental techniques. For example, transient absorption measurements are able to detect non-emissive states. By implementing a similar shuttering technique to what is used in the fluorescence lifetime apparatus, it would be possible to collect transient absorption measurements as a function of illumination time, thus following the evolution of non-emissive states during quenching. Correlating the chlorophyll fluorescence lifetime decays with transient absorption measurements may be able to reveal what states or processes quench chlorophyll. Additionally, isolated thylakoids could be prepared with different amounts of light exposure and cryogenically frozen to preserve the protein arrangement for cryoelectron tomography [177]. This would allow the fluorescence lifetime measurements to be correlated with the protein arrangement. Additionally, models of energy transfer in the thylakoid membrane and of the processes that lead to quenching could be used in combination with the fluorescence lifetime measurements to test hypotheses about the triggering and photophysical mechanisms of quenching.
Bibliography


