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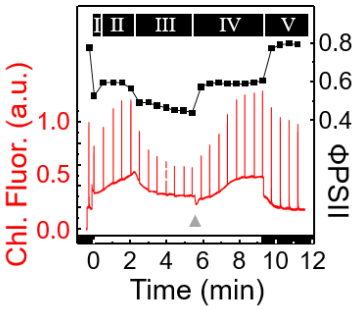

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The author declares they have no conflict of interest.

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Chlorophyll fluorescence of *Chlamydomonas reinhardtii*; insights into the complexities

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Abstract

Tolerance of rapid changes in light intensity by photosynthetic organisms is facilitated by non-photochemical quenching (NPQ), a term with reference to quenching of chlorophyll fluorescence, the technique used in its discovery. Mechanisms of NPQ include dissipating excess light energy to heat (qE), the reversible attachment of light-harvesting complexes (LHC) to photosystems (state transition / qT) and photoinhibition (qI). Chlorophyll is a ubiquitous pigment of photosynthetic organisms, found in LHC and the reaction centres of photosystem II and I (PSII; PSI). At room temperature, pulse-amplitude modulated (PAM) chlorophyll fluorescence protocols provide insights into PSII efficiency, thus a reasonable proxy for photosynthetic activity (carbon fixation), at least under optimal conditions. NPQ has a major impact on chlorophyll fluorescence intensity and is also quantified by PAM. Since NPQ mechanisms can occur simultaneously, they cause complexities in deciphering the signal. In algae, the ability for chlorophyll fluorescence in determining photosynthetic rates is not perfect, but it can still provide valuable information of processes affecting light harvesting. The aim of this report is to provide an overview of how various NPQ mechanisms in the model unicellular chlorophyte alga, *Chlamydomonas reinhardtii*, as well as environmental conditions, affect chlorophyll fluorescence. I also propose a PAM protocol enabling the kinetics associated with each of the NPQ phases to be semi-quantified in under 20 min.

1. Why does photosynthesis need regulating?

Photosynthesis is remarkable. Not only has it lead to all the oxygen that us aerobes need and breathe, it can maximise light use efficiency under highly changeable light intensity, and in accordance with the metabolic demands of the organism. Regulation is coordinated at multiple levels; at the molecular level via energy-transfer processes involving carotenoids and chlorophylls, at the organelle level with supramolecular organization of protein complexes of the thylakoid membrane, at the cellular level by chloroplast relocation, and at the organism level, such as phototaxis and heliotropism. These different types of regulation are of critical importance, enabling photoautotrophy under highly contrasting environments encountered in nature (e.g. suboptimal environment, intermittent light and CO_2 supply). Moreover, regulation prevents damage from absorbance of excess light energy. Chlorophyll is a highly efficient photosensitizer, which in an oxygen-rich environment, which along with spillage of electrons from transfer systems, can generate a damaging level of reactive oxygen species (ROS), leading to photooxidative stress (Roach and Krieger-Liszkay, 2019).

2. Modulating light use efficiency

Light conditions are dynamically fluctuating, requiring light-harvesting systems to be dynamically regulated. Short-term acclimation (seconds to minutes) includes excess energy dissipation (energy-dependent quenching, qE) and energy redistribution of the light-harvesting apparatus (state transitions, qT), which can be activated in response to a low pH of the lumen and reduced redox state of the photosynthetic electron transfer system, respectively, thus forming direct feedback to energetic statuses. Collectively, regulatory mechanisms, such as qE and qT that control how efficiently and where exactly light energy is used (**Fig. 1; Tab. 1**), are known as non-photochemical quenching (NPQ). The term 'quenching' refers to chlorophyll fluorescence, since this is partly how such mechanisms have been discovered and can also be monitored in-vivo (Krause and Weis, 1991). Sustained quenching associated to damaged PSII reaction centres (photoinhibition, qI) is also included in NPQ.

The qE component of NPQ reduces the quantum yield of chlorophyll fluorescence (i.e. induces quenching) in the light-harvesting antennae, and is a pH-dependent process (Müller et al, 2001). It becomes activated by the build-up of protons in the thylakoid lumen, which leads to protonation of light-harvesting complexes (LHC) and absorbed light energy released non-radiatively as heat. In higher plants, and some green algae algae), qE is associated with conversion of violaxanthin to zeaxanthin (Demmig-Adams and Adams, 1996; Quaas et al., 2014; Goss and Lepetit, 2015), an enzymatic process involving violaxanthin de-epoxidase (VDE) and ascorbic acid as substrate (**Fig. 1; No. 2**). Accumulation of protons in the lumen, leading to a pH drop, enable VDE activity, so that excitation energy can be transferred from chl. to zeaxanthin and dissipated as heat.

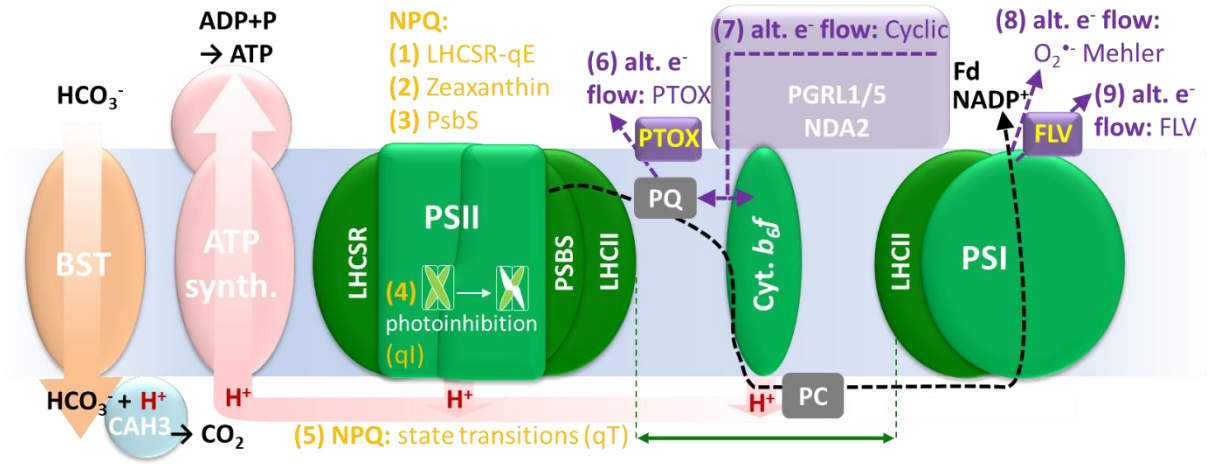

Figure 1. Schematic of protein complexes in the thlylakoid membrane highlighting energy regulation, electron pathways and transport of H^+ and HCO_3^- involved in photosynthesis of *Chlamydomonas reinhardtii*. Black and purple dashed lines denote "linear electron flow" and "alternative electron flow", respectively. See Table 1 for a description of the numbered mechanisms and abbreviations.

Table 1. Light energy regulation, alternative electron pathways and proton translocation in *Chlamydomonas reinhardtii*. The numbers correspond to mechanisms depicted in Figure 1.

Process		Short name	Description	*C. reinhardtii* mutants	Reference
Light energy regulation (NPQ)	1	LHCSR-qE	Main qE mechanism in *C. reinhardtii*, LHCSR3 protects from photo-oxidative stress and fluctuating light	*npq4, lhcsr1*	Peers et al, 2009; Roach et al, 2020
	2	Zeaxanthin	Zeaxanthin accumulation is a ubiquitous high light response. May function in qE	*npq1, npq2*	Niyogi et al, 1997
	3	PsbS	facilitates recruitment of LHCSR, unclear involvement in NPQ	*psbs1*	Correa-Galvis et al, 2016
	4	Photoinhibition (qI)	Light-associated damage to PSII, repairable within hours	*ftsh1-1*	Malnoë et al, 2014
	5	State transitions (qT)	Movement of LHCII between PSII and PSI to balance energy distribution, active during a change in light intensity	*stt7-9, stt7-7, npq4stt7-9*	Depege et al, 2003
Alternative electron pathways	6	PTOX	Plastid terminal oxidase, full reduction of O_2 to H_2O, safety valve	*ptox2*	Houille-Vernes et al, 2011
	7	Cyclic	Reducing power re-invested back into ETC when greater ΔpH for qE or ATP is needed	*pgrl1*	Jokel et al, 2018
	8	ROS	Partial reduction of O_2 to ROS, safety valve	-	-
	9	FLV	flavodiiron, full reduction of O_2 to H_2O before light-induced activation of CO_2 assimilation, safety valve	*flvB*	Chaux et al, 2017

In *C. reinhardtii*, zeaxanthin accumulation is not essential for qE induction and a different reductant than ascorbate is a substrate for VDE (Vidal-Meireles et al, 2020). Instead, NPQ In *C. reinhardtii* is intricately linked to LHC-type Stress-Related (LHCSR) thylakoid membrane proteins, LHCSR1 and LHCSR3 (Figure 1, No. 1; Figure 2), which plants do not have (Peers et al, 2009). Plants and algae possess a PSII-associated protein, PsbS (Figure 1, No. 3), which in plants contributes to qE, but is involved with accumulation of LHCSR (Correa-Galvis et al, 2016; Redekop et al, 2020) Since qE lowers the lifetime of

chlorophyll fluorescence by dissipating the energy as heat, it leads to a detectable quenching of chlorophyll fluorescence (Krause and Weis, 1991; Müller et al, 2001). The governance of qE by pH means that processes affecting proton accumulation in the lumen also affect qE. Protons accumulate in the lumen from water splitting by PSII and electron transfer through the cytochrome b_6f complex (cyt b_6f). Thus, cyclic electron flow (Figure 1, No. 7) that recycles electrons into the electron transfer system also pumps protons into the lumen. Subsequently, ATP synthase uses the protons to convert ADP + P_i to ATP, while conversion of HCO_3^- pumped into the lumen via Bestrophin-Like Proteins (BST) is converted to CO_2 via carbonic anhydrase 3 (CAH3) (Mukherjee et al, 2019), thus also exert influence role on proton concentrations and qE (Figure 1).

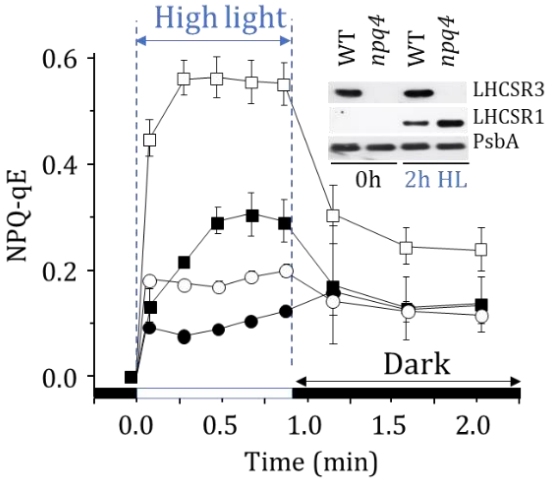

Figure 2. Thermal dissaption (qE) in response to light and dark. LHCSR3-dependent qE rapidly activates in response to high light (HL), while qE capacity increases after prolonged (2h) HL treatment (open symbols), relative to before HL (closed symbols). Using a *C. reinhardtii* mutant deficient in LHCSR3 (*npq4*; circles) the HL-induced increase in NPQ capacity is shown partly due to accumulation of LHCSR1 proteins (inset), that also occurs in wild type (WT) cells (squares). The PSII reaction centre (PsbA) is loading control.

Light energy is also distributed between the two photosystems (PS), PSII and PSI, by movement of LHCII, in a process called state transitions, abbreviated to qT (Figure 1, No. 5). By changing the relative excitation of photosystems, state transitions influence redox poising of the photosynthetic electron transfer system (Rochaix, 2011), and contribute to high light acclimation (Allorent et al, 2013; Bergner et al, 2015). The translocation of LHCII between PSII and PSI is coordinated by its level of phosphorylation (Figure 3). In so-called state 1, LHCII is not phosphorylated and energetically associated with PSII. In response to a reduced plastoquinone pool (PQ) pool, a threo-phosphorylating (protein) kinase (STT7) phosphorylates LHC proteins, in a process involving the cyt b_6f (Dumas et al, 2017). Phosphorylated LHCII migrates to PSI where, in so-called state 2, it can act as antenna for PSI (Rochaix, 2011). Dephosphorylation occurs via a PP2C-type phosphatase when the PQ pool is in an oxidised state. In *C. reinhardtii* up to 80% of LHCII can rapidly disassociate from PSII (Ünlü et al., 2014; Nawrocki et al, 2016), which decreases the level of chlorophyll fluorescence (Allorent et al, 2013; Roach and Na, 2017). Of note, *C. reinhardtii* has a large NADPH-dependent capacity for reducing the PQ pool in darkness (Johnson and Alric, 2012) . This leads to *C. reinhardtii* phosphorylating LHC and inducing state 2 immediately after high light-treated cells are dark-adapted (Figure 3; Roach and Na, 2017).

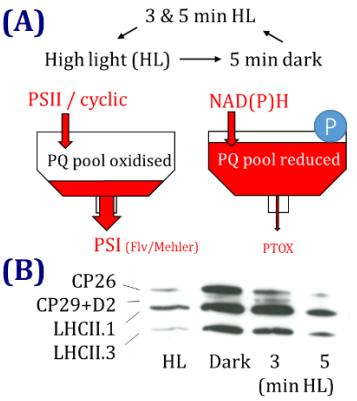

Figure 3. State transitions (qT) in response to light and dark. (A) The electron transfer system visualised as a 'sink' of electrons (e^-). In light, PSII activity pours e^- into the sink, which is emptied by PSI activity (redox state oxidised; state 1). PTOX, FLV and Mehler may also contribute. In the dark, PSII and PSI activity stop, but the sink can fill via Nda2 activtiy, leading to protein phosphorylation (P) of LHC proteins (redox state reduced; state 2). **(B)** Western blot of phosphorylated thylakoid proteins in response to sequential light treatments, from Roach and Na (2017).

3. Photosynthetic electron transfer pathways

The light-driven reaction of water splitting in PSII serves two main purposes: The release of protons and electrons. The build-up of a proton gradient (ΔpH) across the thylakoid membrane is used, much like in mitochondria, to drive ATP synthase conversion of ADP + P_i to ATP, while electrons are used to reduce $NADP^+$ to NADPH (Figure 1). In the first part of the photosynthetic electron transfer system, electrons are passed from PSII to the PQ pool, subsequently to cyt b_6f, and finally via plastocyanin (PC) to PSI, where a second light-driven reaction is used to reduce stromal electron carriers, such as ferreredoxin and eventually $NADP^+$ (Figure 1). Ultimately, ATP and NADPH are mainly used in the Calvin-Benson cycle to assimilate CO_2 into sugars. The additional release of O_2 from water-splitting in PSII also impacts photosynthetic electron transfer, but not directly, rather from photosynthesis operating in an oxidative environment. Oxygen can be used in at least three pathways as an electron-acceptor from the photosynthetic electron transfer system: 1) The plastoquione oxidase (PTOX) oxidises platoquinol to plastoquinone while reducing O_2 to H_2O (Figure 1, No. 6) and thus can poise the redox state of the PQ pool and influence qT (Houille-Vernes et al, 2011); 2) Flavodiirons accept electrons from PSI while reducing O_2 to H_2O (Figure 1, No. 9), and is important when the Calvin-Benson cycle is inactive due to darkness and there is a sudden increase in light intensity, leading to a surge of electrons that cannot be used (Chaux et al, 2017); 3) O_2 can also accept electrons directly in a non-enzymatic process called the Mehler reaction (Figure 1, No. 8), during which the ROS superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced.

Any of the fore-mentioned processes that use O_2 as an electron acceptor avoid the formation of reducing equivalents (i.e. NADPH). Under stress or sub-optimal conditions when CO_2 assimilation is restricted, this can be important to prevent an imbalance of NADPH:ATP and an over-reduced electron transfer system and associated ROS production. When starch synthesis is compromised, a greater proportion of the electrons is directed toward O_2 reduction through both the FLVs and PTOX, suggesting an important role for starch synthesis in priming/regulating Calvin-Benson cycle and electron transfer (Saroussi et al, 2019). Another critical electron transfer process is the so-called cyclic electron pathway (Figure 1, No. 7), either involving proton-gradient regulation (PGR) proteins, (although controversially (Nawrocki et al, 2019; Buchert, 2020), or NAD(P)H:ubiquinone reductase 2 (NDA2), proteins (Jans et al, 2008). Importantly, photoreduction of O_2, and especially cyclic electron pathway, promote ATP formation by using the light-driven reactions to create a ΔpH. Specifically relevant to algae, low CO_2

availability in the water-column has resulted in the evolution of carbon-concentrating mechanism (CCM) for RUBISCO, the first enzyme in carbon fixation, to efficiently access its substrate CO_2. In H_2O, CO_2 dissolves into carbonic acid that disassociates to bicarbonate HCO_3^- and carbonate CO_2^{2-}, which can be converted back to CO_2 in an acidic environment via CAH3 (Figure 1). Thus, the combined action of cyclic electron flow and O_2 photoreduction generate a low luminal pH is essential for CCM function (Burlacot et al, 2022).

4. A very brief guide to the origin of chlorophyll fluorescence

Pulse-amplitude modulated (PAM) chlorophyll fluorescence is a non-invasive measurement of PSII activity. It can be used to calculate linear photosynthetic electron transfer rates towards an estimation of carbon assimilation, as well as an invaluable indicator of organism 'health' state. Within the thylakoid membrane, LHC that are also called 'antenna', facilitate in capturing light energy and its transfer to the reaction centres (PSII and PSI) to drive photochemistry (Figure 1). When light arrives at LHC it has one of three fates, 1) it can be passed to the PS for powering the light reactions of photosynthesis, 2) it can be released as heat (i.e. thermal dissipation, including qE), and 3) a few % will always be released as fluorescence, an inherent property of chlorophyll. Thus, fluorescence intensity is influenced by the light intensity. It is also intricately linked to the redox state of the electron transfer system, because when electrons can no longer be passed from PSII into the electron transfer system (i.e. it is already fully reduced) more energy is released as fluorescence. This is how photosynthesis can also be probed. For a review see (Maxwell and Johnson, 2000). Photosynthesis-saturating pulses of light are repeatedly applied (e.g. 20 s intervals) that fully reduce the transfer system and lead to maximum fluorescence (F_m), evident by the fluorescence spikes in Figure 4. The ratio between F_m and minimum fluorescence just before the pulse (F_o in dark; F_t in light) is used to calculate the quantum efficiency of PSII, which in the dark is at a maximum (so-called F_v/F_m), and lower in the light (so-called ΦPSII). The ΦPSII changes depending on CO_2 availability and NPQ, as shown in a PAM quenching kinetic of photoautotrophic *C. reinhardtii* wild type cells (Figure 4).

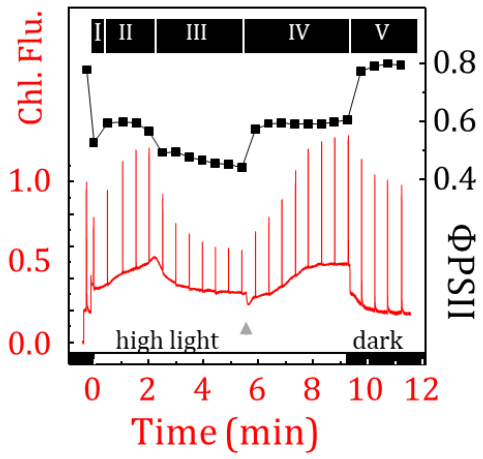

Figure 4. Effect of light-induced CO_2 depletion on NPQ. Placing high light acclimated *C. reinhardtii* cells into dark induces qT state 2 (Figure 3) and LHCII to decouple from PSII, decreasing F_m. Re-treating cells with high light (white bar, X axis), causes transition to state 1 (Figure 3) and LHCII to couple with PSII, increasing F_m (red spikes; 0-2min), as well as activation of qE (F_m quenching, 2-5min) as CO_2 depletes. 1mM HCO_3^- was added just before 6min (grey arrow) relaxing qE. Effects on ΦPSII are shown (squares, right Y-axis) and corresponding NPQ phases I to V (top black boxes) are described in the text.

5. Overlapping NPQ mechanisms confound chlorophyll fluorescence measurements

NPQ is calculated from a chlorophyll fluorescence analysis by $(F_m°-F_m')/F_m'$, with $F_m°$ and F_m' measured in before (usually dark-acclimated) and during (e.g. in light) treatment, respectively. Thus, quenching of F_m is indicative of NPQ. In Figure 4, the change in F_m value reveals five distinct NPQ phases during a dark-light-dark treatment of pre-high light acclimated *C. reinhardtii* cells. These phases have been resolved using mutants deficient qE (i.e. *npq4*) and qT (i.e. *stt7*) (Allorent et al, 2013; Roach and Na, 2017), and are (I) immediate qE of state II cells, (II) qT transition from state II to I, (III) continued induction of qE of state 1 cells as CO_2 deplates, (IV) relaxation of qE, in this case after HCO_3^- was added, and (V) qT transition from state I to II of cells in the dark (Figure 4). When photosynthesis is hindered due to low CO_2 availability, activation of qE lowered ΦPSII since an increase in light energy is partitioned to thermal dissipation rather than driving photochemistry. At room temperature, the level of fluorescence from PSI and associated antenna is lower and is often disregarded, although for accurately calculating electron transport rates needs consideration (Pfündel et al., 2013). At much colder temperatures (e.g. at 77K; –196°C), biochemical and physiological processes that modulate chlorophyll fluorescence are mostly abolished, and the fluorescence emission of both PSI and PSII is distinguishable. An aliquot of cells measured in Figure 4 were also removed for analysis at 77K (Figure 5). Here, it becomes obvious that the ratio of fluorescence originating from PSII and PSI changes throughout the treatment as qE activates and relaxes, and qT transitions between states 1 and 2.

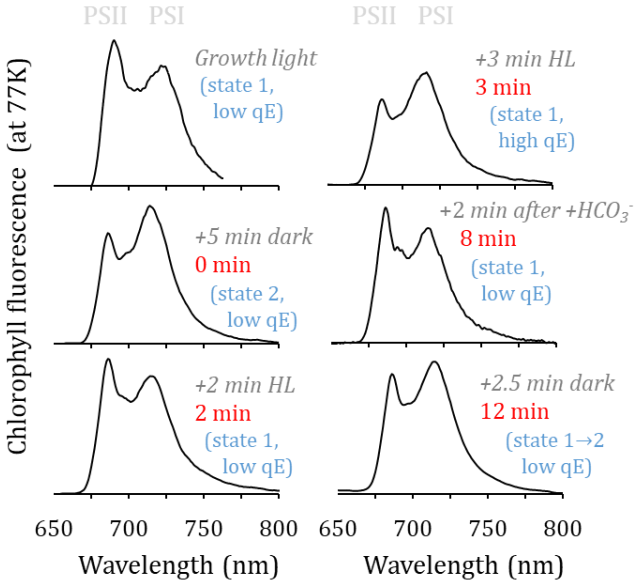

Figure 5. Effect of light treatments and CO_2 depletion on chlorophyll fluorescence measured at 77K. Fluorescence peaks at 685 and 715nm correspond to PSII and PSI emission, respectively (excitation at 440nm). Samples were from cells treated in Figure 4 at 0, 2, 3, 8 and 12 min (red), with major influence of most recent treatment (italic grey), and NPQ state (blue) given for each measurement.

Although different aspects of NPQ are regulated by distinct mechanisms (Table 1), it known that individual process affect others, such as transition to state 1 increasing antenna size inducing a more rapid activation of qE (Allorent et al, 2013), and LHCSR3 also having involvement in qT (Roach and Na, 2017). Further complexity arises from temporal overlap between, e.g. NPQ phases I-III activated in response to light, which each have opposing effects on fluorescence. Similarly, if HCO_3^- had not been added to cells in Figure 4, qE would have relaxed later in the dark (increasing F_m as in Figure 6A) while cells transition to state 2 (decreasing F_m). What also becomes obvious from such analyses is that the light acclimation state of cells has a significant impact on NPQ phases. For examples, not only does the qE capacity increase in high light-acclimated cells (Figure 2), but qT is also amplified (Figure 6A). This may involve a change in rates of activity of processes described in Table 1. It can be shown in various NPQ mutants that the increase in F_m between 0.5-3 min (NPQ phase II; Figure 4), attributed to transition to state 1, consistently shows a positive relationship with the level qE after 10 min high light (NPQ phase III (Figure 4), supporting that the qE protein in *C. reinhardtii*, LHCSR3, has a role in qT (Figure 6B). In agreement with this, some increase in F_m also occurred in *stt7-9* (Figure 6B), indicating processes in addition to LHC de-phosphorylation are also involved in NPQ phase II.

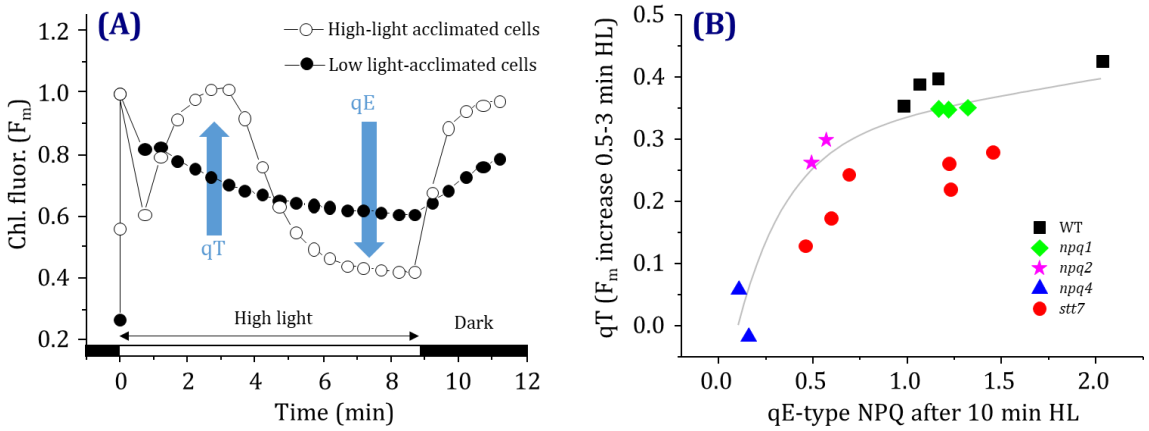

Figure 6. Effect of light-acclimated state on activation of NPQ processes. (A) *C. reinhardtii* photoautotrophic cells were either low or high light-acclimated and dark adapted for 15 min before measuring chlorophyll fluorescence at 450 µmol photons m^{-2} s^{-1} (high light) and darkness. Symbols are of F_o at 0 min, and F_m thereafter (both normalised to F_m°), with influence of qT transition to state 1 and qE activation depicted with blue arrows. (B) Correlation of F_m increase between 0.5 and 3 min after dark-to-high light treatment, with qE after 10 min high light, in *C. reinhardtii* wild type (WT) and various NPQ mutants (see Table 1) high light-acclimated for between 0-4h.

Accelerated metabolism in high light-acclimated cells and cross-organelle metabolite exchange, potentially leading to more rapid fluctuations in NADPH and ATP levels, no doubt also contribute to energy regulation, although our understanding of such short-term metabolism at sub-cellular level is poor. Coupling non-invasive measurements, such as chlorophyll & NAD(P)H fluorescence, and net O_2 flux, with profiling of primary

metabolites will provide a more holistic understanding of how photosynthesis responds to changes in light intensity.

6. A protocol for semi-quantifying NPQ phases with PAM chlorophyll fluorescence in liquid *C. reinhardtii* cultures

Considering the potential complexity of deciphering PAM chlorophyll fluorescence of *C. reinhardtii*, there is need for a protocol that allows distinguishing the individual NPQ components. In Figure 7, cells have been treated in specific sequence of light treatments of defined duration (Figure 7) so that kinetics associated with each of the NPQ phases (in brackets), introduced in Figure 4, can be semi-quantified in under 20 min in a photoautotrophic liquid culture: **0-0.5 min** (I: immediate qE), **0.5-2 min** (IIa: qT transition to state 1a), **3-5 min** (IIb: qT transition to state 1b), **5-7 min** (III: CO_2 depletion-induced qE), **9-10 min** (IV: qE relaxation), **11-13 min** (V: qT transition to state 2), and an additional phase **14-18 min** (VI: qT transition to state 1) in response to far-red light under which only PSI activity occurs and the PQ pool oxidises (Figure 4). For calculating qE, the F_m measured at the end of far-red light treatment when cells are in state 1 should be used rather than F_m before actinic light treatment when cells are in state 2, otherwise qE will be underestimated.

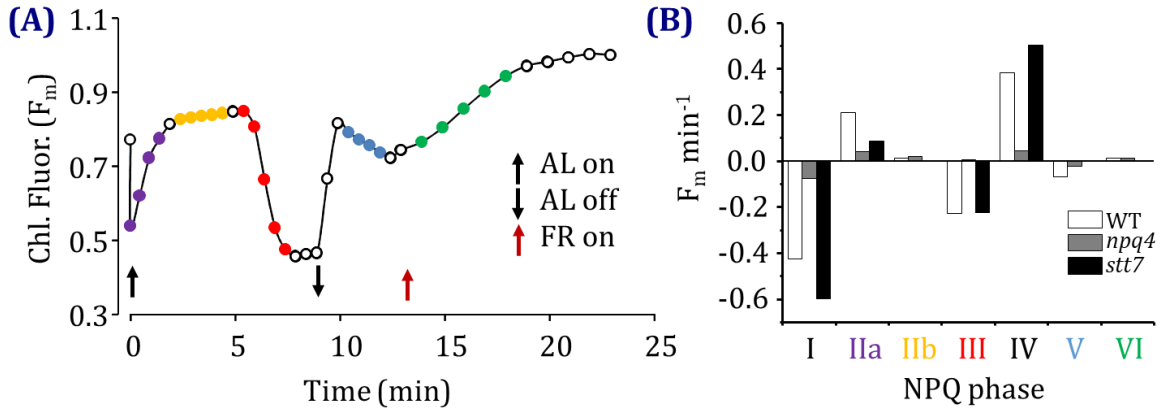

Figure 7. A PAM protocol to semi-quantify NPQ phases. (A) Changes in F_m in response to actinic light (AL on) of 450µmol photons m^{-2} s^{-1}, darkness (AL off), and far red light (FR on) of maximum intensity using a PAM 2500 (Walz). 2mL of wild type *C. reinhardtii* culture at 7.5µg chlorophyll mL^{-1} and acclimated to 250µmol photons m^{-2} s^{-1} was in a 3mL cuvette and dark-treated for 15min before starting, and constantly stirred with a mini rotating magnetic during measurement. Saturating pulses were every 30s, or 2min in FR. Colour symbols and NPQ phases are given in text. (B) Kinetics of each NPQ phase calculated for WT, *npq4* and *stt7-9* expressed as change in F_m min^{-1}.

For proof of concept of the PAM protocol and confirmation of NPQ phases, the *npq4* and *stt7-9* mutants were also measured in the same way (Figure 7B), showing that NPQ phase I, IIa, III and IV are heavily influenced by LHCSR3, while IIb, V and VI are STT7-dependent, as reported by Roach and Na (2017). It is worth considering that the longer cells are dark-treated before measurement increases the duration of phase IIb (Roach and Na, 2017). Furthermore, the intensity of actinic light during measurement also has

an influence, with a higher light intensity activating qE earlier and reducing the time before induction of phase III. The density of culture also has a major influence, not only on the intensity of chlorophyll fluorescence signal, but also on how quickly CO_2 depletes from the media, and how much self-shading occurs within the culture. Finally, if cells have been high light-treated before measuring, then recovery from qI may also lead to a gradual increase in F_m over time as PSII is repaired. This has implications for calculating NPQ, since reference is typically made to the first F_m before light treatment during fluorescence measurements.

Abbreviations

BST	Bestrophin-Like Proteins,
CAH3	Carbonic anhydrase 3
cyt b_6f	Cytochrome b_6f complex
Fd	Ferredoxin
FLA	Flavodiiron
F_m/F_o	Maximum/Minimum chlorophyll fluorescence
LHC	Light-haversting compex
LHCSR	LHC-stress-related protein
NPQ	Non-photochemical quenching
PAM	Pulse-amplitude modulated
PC	Plastocyanin
ΔpH	Trans-membrane proton gradient
ΦPSII	Relative quantum yield of PSII
PsbS	Subunit S of PSII
PSII/PSI	Photosystem II/I
PQ	Plastoquinone
LHC	Light-haversting compex
LHCSR	LHC-stress-related protein
NPQ	Non-photochemical quenching
qE	Thermal dissipation-type NPQ
qI	Photinhibition-type NPQ
qT	State transition-type NPQ
ROS	Reachtive oxygen species
STT7	STT7 kinase

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