

1 **Interplay between LHCSR proteins and state transitions governs the NPQ response in**
2 **intact cells of *Chlamydomonas* during light fluctuations.**

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24 A.H.S. performed research; C.J.S., A.B., A.H.S. and G.R.F. analyzed data; C.J.S. and A.B.
25 wrote the paper with inputs from A.H.S., K.K.N. and G.R.F.

26

27 **One sentence summary:** The roles of LHCSR and STT7 in NPQ vary with the light
28 fluctuation period and duration of light fluctuation.

29

30 **Key words:** photoprotection, non-photochemical quenching, chlorophyll fluorescence,
31 bioenergetics, microalgae, light-harvesting complex stress related, state transition.

32 **Abstract**

33 Photosynthetic organisms use sunlight as the primary energy source to fix CO₂. However, in
34 the environment, light energy fluctuates rapidly and often exceeds saturating levels for
35 periods ranging from seconds to hours, which can lead to detrimental effects for cells. Safe
36 dissipation of excess light energy occurs primarily by non-photochemical quenching (NPQ)
37 processes. In the model green microalga *Chlamydomonas reinhardtii*, photoprotective NPQ is
38 mostly mediated by pH-sensing light-harvesting complex stress-related (LHCSR) proteins and
39 the redistribution of light-harvesting antenna proteins between the photosystems (state
40 transition). Although each component underlying NPQ has been documented, their relative
41 contributions to the dynamic functioning of NPQ under fluctuating light conditions remains
42 unknown. Here, by monitoring NPQ throughout multiple high light-dark cycles with
43 fluctuation periods ranging from 1 to 10 minutes, we show that the dynamics of NPQ depend
44 on the frequency of light fluctuations. Mutants impaired in the accumulation of LHCSRs
45 (*npq4*, *lhcsr1*, and *npq4lhcsr1*) showed significantly less quenching during illumination,
46 demonstrating that LHCSR proteins are responsible for the majority of NPQ during repetitive
47 exposure to high light fluctuations. Activation of NPQ was also observed during the dark
48 phases of light fluctuations, and this was exacerbated in mutants lacking LHCSRs. By
49 analyzing 77K chlorophyll fluorescence spectra and chlorophyll fluorescence lifetimes and
50 yields in a mutant impaired in state transition, we show that this phenomenon arises from state
51 transition. Finally, we quantified the contributions of LHCSRs and state transition to the
52 overall NPQ amplitude and dynamics for all light periods tested and compared those with cell
53 growth under various periods of fluctuating light. These results highlight the dynamic
54 functioning of photoprotection under light fluctuations and open a new way to systematically
55 characterize the photosynthetic response to an ever-changing light environment.

56 **Introduction**

57 Most life on Earth is sustained by photosynthetic organisms that capture sunlight energy to
58 convert CO₂ and water into chemical energy. Light is captured by light-harvesting antenna
59 complexes that contain a network of pigments absorbing photons and funneling the energy
60 towards photosystems II and I that use it to perform photochemical reactions. Under light-
61 limiting conditions, efficient light harvesting is crucial for maximizing the rate of CO₂
62 fixation (Björkman and Demmig, 1987). However, high light (HL) intensities can saturate
63 reaction centers and lead to the build-up of excess excitation energy, which, if unchecked, can
64 lead to the production of reactive oxygen species and damage to both reaction centers
65 (Khorobrykh *et al.*, 2020). In nature, light exposure rapidly fluctuates in intensity with periods
66 of HL ranging from milliseconds to hours (Graham *et al.*, 2017), requiring photosynthesis to
67 acclimate to different frequencies of HL fluctuations. For each period of HL acclimation,
68 photosynthetic organisms exhibit photoprotective mechanisms that regulate light harvesting
69 and safely remove excess energy (Erickson *et al.*, 2015; Pinnola and Bassi, 2018; Roach,
70 2020).

71 Upon light absorption, the energy can be dissipated as heat in a process called non-
72 photochemical quenching (NPQ). NPQ involves five components, each of which has been
73 distinguished by its time of induction and relaxation during transition between dark and HL
74 (Erickson *et al.*, 2015). The fastest component, called energy-dependent quenching (qE), is
75 triggered by luminal acidification (Briantais *et al.*, 1979) and is induced and relaxed within
76 seconds. State transition (qT) occurs within minutes and involves the phosphorylation of
77 light-harvesting complexes (LHCs) (Allen, 1992) resulting in their detachment from
78 Photosystem (PS) II and subsequent aggregation in a quenched state and/or association to PSI
79 (Nagy *et al.*, 2014; Ünlü *et al.*, 2014; Nawrocki *et al.*, 2016). Zeaxanthin-dependent
80 quenching (qZ) requires the accumulation of zeaxanthin and probably involves quenching in
81 the minor LHCs of PSII (Dall'Osto *et al.*, 2005; Wehner *et al.*, 2006; Nilkens *et al.*, 2010). On
82 longer time scales, two more sustained forms of NPQ occur: qH that takes places in the
83 antennae of PSII (Malnoë *et al.*, 2018) directly in the LHCII trimers (Bru *et al.*, 2021) and
84 photoinhibition (qI) that occurs when degradation of the PSII core protein D1 exceeds its
85 capacity for repair due to excess excitation energy (Aro *et al.*, 1993).

86 In the green microalga *Chlamydomonas reinhardtii*, qE is mediated by pigment-binding LHC
87 stress-related (LHCSR) proteins (Peers *et al.*, 2009; Rochaix and Bassi, 2019). LHCSRs
88 contain protonatable residues, which sense the decreasing luminal pH generated under HL
89 conditions (Ballottari *et al.*, 2016; Tian *et al.*, 2019); the protonation of LHCSRs triggers

90 NPQ within the protein (Liguori *et al.*, 2013; Kondo *et al.*, 2017; Troiano *et al.*, 2021),
91 allowing fast activation of qE. While there are two types of LHCSR proteins (LHCSR1 and
92 LHCSR3), both of which bind pigments (Bonente *et al.*, 2011; Perozeni *et al.*, 2020),
93 LHCSR3 (for which two homologs are present in *Chlamydomonas*) is thought to be the main
94 actor in qE (Peers *et al.*, 2009; Truong, 2011). On the other hand, qT is activated by the
95 buildup of reducing equivalents in the thylakoid membrane, which activates a
96 serine/threonine-protein kinase (STT7) (Depege *et al.*, 2003; Lemeille *et al.*, 2009) that
97 phosphorylates LHCII, enabling it to detach from PSII and ultimately reattach to PSI (Iwai *et al.*,
98 2010a; Minagawa, 2011). While qZ has been described in *Chlamydomonas* (Niyogi *et al.*,
99 1997), it does not seem to play a significant role in overall NPQ (Girolomoni *et al.*, 2019;
100 Tian *et al.*, 2019), and its potential mechanism of action remains to be determined. Finally,
101 while qH has not been described in *Chlamydomonas*, qI occurs upon continued excess
102 illumination (Aro *et al.*, 1993; Erickson *et al.*, 2015) at the level of the PSII center, where
103 oxygen-mediated sensitization creates the irreversible formation of a quenching site
104 (Nawrocki *et al.*, 2021).

105 The photophysical and biochemical bases for NPQ have been studied for decades (Erickson *et al.*,
106 2015), however the *in vivo* operation has mostly been assessed under a single dark-to-HL
107 transition (Nedbal and Lazár, 2021) leaving our understanding of photoprotection under more
108 complex light patterns limited. While LHCSR and STT7 activity are both known to be
109 important for steady-state NPQ under prolonged/continuous illumination (Allorent *et al.*,
110 2013), their relative contributions to NPQ have not been quantified, and their response to
111 faster-timescale fluctuating light remains unstudied. Recent work has started looking at the
112 response of NPQ to some specific light fluctuations in *Chlamydomonas* (Roach, 2020) and in
113 the moss *Physcomitrella* (Gao *et al.*, 2021). However, the physiological role and the
114 functioning of the NPQ components under the wide diversity of light patterns that are present
115 in the natural environment is unexplored.

116 Here we utilized two distinct methods to monitor chlorophyll fluorescence in intact cells of
117 *Chlamydomonas* that were exposed to varying frequencies of fluctuating light with HL/dark
118 periods ranging from 1 to 10 min (**Fig. 1**). The roles of qE and qT were investigated using
119 single or double mutants of LHCSRs and STT7. Our analysis of LHCSR mutants (*npq4*
120 (Peers *et al.*, 2009), *lhcsr1* (Truong, 2011), and *npq4lhcsr1* (Truong, 2011)) revealed that
121 LHCSR3 is the main contributor to the NPQ response during the HL phase of light
122 fluctuations. Using mutants impaired in state transition (*stt7* (Depege *et al.*, 2003) and
123 *stt7npq4* (Allorent *et al.*, 2013)), we showed that qT quenching occurs primarily during the

124 dark portion of the fluctuating light sequence and represents a significant part of NPQ during
125 repeated light fluctuations. Our results showed that while qE and qT sustain most of the NPQ
126 throughout light fluctuations, their relative importance varies during different phases of the
127 fluctuating light response, with qT playing a larger role during dark periods and after repeated
128 HL-dark fluctuations. Surprisingly, the light fluctuation period did not seem to have a major
129 impact on the respective contributions of qE and qT although the contribution of qE during
130 the dark phase was period dependent. Nonetheless, the various components of NPQ are not
131 completely independent, and there may be an interplay between LHCSR- and STT7-mediated
132 NPQ that enables the wild-type photoprotective response. We further show that while *stt7*
133 mutants are not impaired in growth under light fluctuations, short time scale light fluctuations
134 highly impair LHCSR mutants. These findings represent an important first step in
135 investigating the photosynthetic response to the diversity of HL periods that occur in nature.

136

137 **Results**

138 **Varying light fluctuation periods affect the dynamic NPQ response.**

139 While the photosynthetic response of *Chlamydomonas* to some light fluctuations has been
140 reported (Cantrell and Peers, 2017; Roach, 2020), an analysis of NPQ for various periods of
141 light fluctuations is lacking. We therefore measured chlorophyll fluorescence during light-
142 dark cycles with fluctuation periods ranging from 1 min to 10 min (**Fig. 1**). Chlorophyll
143 fluorescence yield was measured using pulse-amplitude modulated (PAM) fluorometry and
144 used to calculate NPQ (Klughammer and Schreiber, 2008). In tandem experiments, time-
145 correlated single photon counting (TCSPC) was used to measure the chlorophyll fluorescence
146 lifetime (Amarnath *et al.*, 2012), which was used to calculate NPQ τ (Sylak-Glassman *et al.*,
147 2014). For all periods of light fluctuations in the wild-type strain, NPQ quickly turned on
148 upon illumination but turned off more slowly (**Fig. 2, Supp Fig. 1**). The same trend was
149 observed in NPQ τ (**Fig. 2**). The 1 min period light fluctuation led to a nearly square-like
150 response of NPQ and NPQ τ (**Fig. 2**).

151 For the longer fluctuation periods ranging from 2 minutes to 10 minutes, after an initial burst
152 of NPQ in HL, the level of NPQ decreased with continued illumination, eventually reaching a
153 steady state for the 10 min fluctuating period (**Fig. 2**). A similar trend was also seen in NPQ τ
154 (**Fig. 2**). Therefore, these kinetics are directly related to chlorophyll fluorescence quenching,
155 rather than other non-quenching processes that affect chlorophyll fluorescence. This
156 phenomenon of decreasing NPQ during the light has been previously attributed to the

157 consumption of the proton gradient by the activity of the CO₂ concentration mechanism
158 (CCM) (Burlacot *et al.*, 2021). For fluctuating light periods longer than 4 minutes, upon a
159 transition from HL to dark, the NPQ turned off rapidly but was then followed by a gradual
160 rise in NPQ during further darkness, a trend that was also observed in NPQ_τ (**Fig. 2**).
161 However, compared to NPQ, NPQ_τ showed a larger magnitude of increase during the long
162 dark periods (**Fig. 2C,D**). Differences between the NPQ and NPQ_τ traces are considered in
163 the discussion.

164 We conclude from these experiments that, when exposed to light fluctuations with periods
165 ranging from 1 to 10 min, at least three components of NPQ are present: (i) a rapidly
166 responding component, (ii) a slowly inducible component induced throughout the light
167 fluctuations, and (iii) a component induced in the dark phases of light fluctuations.

168

169 **The majority of NPQ during light fluctuations is mediated by LHCSR proteins.**

170 It has been well established that LHCSR proteins are crucial for NPQ in *Chlamydomonas*
171 during a single dark-to-light transition (Peers *et al.*, 2009; Truong, 2011; Correa-Galvis *et al.*,
172 2016). To examine the relative importance of each LHCSR protein for the functioning of
173 NPQ during light fluctuations, we measured the chlorophyll fluorescence yield and lifetime
174 during the same light-dark cycles on mutants impaired in the accumulation of LHCSR1
175 (*lhcsr1*) (Truong, 2011), LHCSR3-1 and LHCSR3-2 (*npq4*) (Peers *et al.*, 2009), or all three
176 LHCSRs (*npq4lhcsr1*) (Truong, 2011; Ballottari *et al.*, 2016). While the *npq4lhcsr1* mutant
177 was highly impaired in its NPQ capacity for all light fluctuations (**Fig. 2, Supp Fig. 1**), single
178 *npq4* and *lhcsr1* mutants showed some NPQ in response to light fluctuation (**Fig. 3, Supp**
179 **Fig. 2**). Noticeably, for fluctuating periods longer than 4 minutes, the increasing NPQ
180 observed during dark phases was more pronounced in the *npq4lhcsr1* mutant (**Fig. 2**). We
181 conclude from these experiments that although LHCSRs are responsible for most of the NPQ
182 during the light phase of all light fluctuations, a substantial portion of the NPQ in WT is
183 nonetheless mediated by other biochemical processes, part of which is induced during the
184 dark periods of light fluctuations.

185

186 **The increasing quenching in the dark periods arises from state transition**

187 Induction of NPQ during darkness has been previously reported in chlorophytes (Casper-
188 Lindley and Björkman, 1996; Allorement et al., 2013), and qT has been proposed to be involved
189 (Allorement et al., 2013). Re-organization of light-harvesting antennae between PSII and PSI
190 was thus followed throughout a light fluctuation by measuring 77K fluorescence emission
191 spectra. The spectra for cells were compared at three time points: after acclimation to far-red
192 light (cells in state 1 (Zhang *et al.*, 2021)), after 10 min HL, and after 10 additional min dark
193 (see arrows/lines in **Fig. 4**). In WT and *npq4lhcsr1*, an increase in the emission at 710 nm
194 specific to PSI-bound LHCII was observed between the 10 min (after HL) and 20 min (after
195 dark) time points, suggesting that some re-association of LHCII from PSII to PSI occurs
196 during the dark portion of our measurements (**Fig. 4, Supp Fig. 3**). In contrast, mutants
197 lacking the STT7 kinase responsible for qT (*stt7* and *stt7npq4*) showed negligible changes in
198 the 77K fluorescence emission spectra (**Supp Fig 4**) and only showed a minimal increase in
199 NPQ or NPQ τ during the dark periods of light fluctuations (**Fig. 5, Supp Fig. 5**) and with
200 increasing duration of exposure to light fluctuations (**Supp Fig. 6**).

201 To characterize the kinetics of qT occurring during the light-to-dark transition, we analyzed
202 the response of the chlorophyll fluorescence lifetime for WT and *npq4lhcsr1* mutant cells that
203 were exposed to 10 min of HL followed by 30 min of dark. Interestingly, upon light-to-dark
204 transition, both strains showed steadily decreasing lifetimes for the first 10 min of darkness,
205 after which, the fluorescence lifetimes began to reverse, eventually reaching the starting
206 lifetime after 30 minutes of darkness (**Supp Fig. 7**). Therefore, we conclude that the
207 quenching observed during the dark phases of light fluctuations in *Chlamydomonas* arises
208 from qT, which has an induction timescale of ~10 min and is reversible upon continued
209 exposure to darkness.

210

211 **Quantification of NPQ vs growth under fluctuating light.**

212 It has been previously proposed that, while LHCSRs play an important role during short
213 periods of illumination, state transitions are important for longer periods of high light
214 acclimation (Erickson et al., 2015). To test this hypothesis and assess the scenario under
215 fluctuating light conditions, we quantified the amount of NPQ that was mediated by each
216 protein by comparing the remaining NPQ (or NPQ τ) in each mutant relative to the NPQ (or
217 NPQ τ) in the WT reference strain. Surprisingly, the contribution of each protein to overall
218 NPQ did not seem to depend on the period of the light fluctuation (**Supp Fig. 8**). While

219 LHCSR3 is responsible for the majority of overall NPQ (72%, **Fig. 6B**), STT7 had a
220 substantial contribution mediating 42% of the NPQ, with LHCSR1 having a smaller
221 contribution at 22% of NPQ. LHCSRs were found to have a substantially larger contribution
222 during light phases, where they are responsible for 94% of WT NPQ, while in the dark
223 phases, their contribution declined to 57% (**Fig. 6, Supp Fig. 9**). On the other hand, STT7
224 played a significantly larger role in the NPQ during darkness (60%) than it does during
225 illumination (36%). Interestingly, the amount of NPQ mediated by LHCSRs was more
226 important during the beginning of light fluctuations while state transitions contributed more
227 after 20 minutes of light fluctuation (**Fig. 6, Supp Fig. 10**), revealing increased relative
228 contribution of qT and decreasing contribution of qE with increasing time of exposure to light
229 fluctuations.

230 Although mutants impaired in the accumulation of LHCSRs and STT7 have strongly impaired
231 NPQ capacities, this does not seem to impair the growth of those strains under continuous
232 high light conditions (Depege et al., 2003; Peers et al., 2009; Cantrell and Peers, 2017).
233 Recent data have shown that the growth of *npq4* and *npq4lhcsr1* mutants is impaired under
234 certain light fluctuation conditions (Cantrell and Peers, 2017; Roach, 2020). We therefore
235 investigated whether the growth impairment of those strains could be dependent on the period
236 of light fluctuation. While all the mutants grew as well as the control strain under continuous
237 illumination (**Supp. Fig. 11**), *npq4*, *lhcsr1*, *npq4lhcsr1* and *st7npq4* mutants exhibited
238 impaired growth under fast light fluctuations with a 1-minute period (**Fig. 7**). In contrast, only
239 *npq4lhcsr1* and *st7npq4* mutants showed an impaired growth under slower light fluctuations
240 with a period of 10 minutes, and the growth of all mutants was similar when the period was
241 increased to 30 minutes (**Fig. 7**). We conclude from this experiment that qE mediated by
242 LHCSR proteins is critical for growth under light fluctuations and that this role is more
243 important for short light fluctuations.

244

245 **Discussion**

246 The involvement of pH-sensing LHCSR proteins and state transitions in the photoprotective
247 response of *Chlamydomonas* has been previously described (Peers et al., 2009; Allore et al.,
248 2013). While mutants impaired in accumulation of LHCSRs were shown to have limited
249 growth when light is provided in a day/night cycle (Cantrell and Peers, 2017) or fluctuating
250 with a 10-minute period (Roach, 2020), our understanding of the contribution of LHCSRs and

251 state transitions to photoprotection during fluctuating light remains limited. Here, by
252 measuring the NPQ levels during light fluctuations in a range of mutants impaired in the
253 accumulation of LHCSR3, LHCSR1, and/or STT7 we have unraveled their relative
254 contributions to NPQ. Varying the length of fluctuating periods from 1 to 10 min allowed us
255 to assess the dynamics of rapid qE- and slower qT-type processes. Interestingly, we observe
256 that qT builds up during the dark periods of the light fluctuations and continues to play a role
257 in the NPQ response during subsequent light phases. It occurs even in the absence of
258 LHCSR3 (see *npq4* and *npq4lhcsr1* mutant in **Fig. 2** and **Fig. 4**), has a timescale of 10 min,
259 and is reversible (Supp **Fig. 7**), which is consistent with recent literature (Allorent et al., 2013;
260 Zhang et al., 2021). During a transition between low-light and high-light stress, qE proteins
261 take a few hours to be fully induced (Peers et al., 2009), and it was hypothesized that qT may
262 substitute for qE during this delay (Allorent et al., 2013). Our results show that even when
263 LHCSRs are fully activated (i.e., in high light-acclimated cells), the occurrence of qT remains
264 substantial during light fluctuations (**Fig. 2**). State transition or qE mutants were previously
265 shown to have high reactive oxygen species (ROS) production (Allorent et al., 2013; Barera *et*
266 *al.*, 2021). Thus, the substantial amount of qT induced during the dark periods of light
267 fluctuations may enhance photoprotection and limit ROS production by “anticipating” the
268 next exposure to high light. The combination of fast qE (turns on rapidly upon HL exposure
269 due to ΔpH) and residual qT (from previous dark periods) could therefore provide effective
270 photoprotection in an unpredictable fluctuating-light environment.

271

272 Since qE has long been ascribed as the fastest component of NPQ, directly responding to the
273 thylakoid lumen proton concentration (Briantais et al., 1979), and qT as a slower component
274 (Allorent et al., 2013), the contribution of qE to NPQ was proposed to be stronger for short
275 periods of HL, with the contribution of qT becoming increasingly important during longer
276 periods of high-light stress (Erickson et al., 2015). Our approach of systematically assessing
277 the response of photosynthesis to various periods of light fluctuations has revealed nuances in
278 this interpretation. Surprisingly, we found that the overall contributions of qE and qT were not
279 dependent on the period of light fluctuations tested (**Fig. 6** and **Supp. Fig. 8**). However, the
280 *npq4* mutant showed significantly reduced NPQ capacity compared to WT in the dark periods
281 (by 74%) under fast light fluctuations (1-1 sequence), but only a 17% impairment under
282 slower fluctuations (10-10) (see **Supp. Fig. 9**), showing that relaxation of qE (around 1 min)
283 is mediated by LHCSR3 and contributes substantially to the response of NPQ to short periods
284 of light fluctuations. Such relaxation kinetics may contribute to a faster response of NPQ to

285 the next illumination if the period of light fluctuations is shorter than 2 minutes. It is also
286 worth noting that the relative importance of qE in NPQ decreased after 20 minutes of light
287 fluctuations, while the opposite occurred for qT (**Fig. 6**), reflecting a build-up in qT
288 throughout the 40 minutes of light fluctuations. Modeling the response of photosynthesis to
289 complex light fluctuations has been done (Zaks *et al.*, 2012; Zaks *et al.*, 2013; Tanaka *et al.*,
290 2019; Steen *et al.*; Nedbal and Lazár, 2021) and would allow targeting specific mechanisms
291 for increasing plant yields in the field. Our results show that such efforts should consider both
292 the period of light and dark as well as the total time exposed to fluctuating light. In green
293 microalgae it is tempting to speculate that in nature, where exposure to HL and dark occur
294 repeatedly, qE may play a more important role in the beginning of light fluctuations while qT
295 may provide a photoprotective response on a longer time scale.

296

297 Interestingly, when comparing NPQ and NPQ τ , the magnitude of the quenching decrease
298 during HL was larger for NPQ τ than NPQ (**Fig. 2**). The energetic requirement (and thus its
299 proton gradient consumption) of the CCM depends on the inorganic carbon (C_i) availability
300 (Fei *et al.*, 2021). Since the high cell concentration in the TCSPC sample leads to strong C_i
301 consumption, this could deplete the C_i concentration even in the presence of bubbling, leading
302 to a C_i level sensed by cells in the TCSPC sample being lower than what is experienced by
303 cells in the PAM sample. This would lead to higher activity of CCM, and hence a larger
304 decrease in quenching, under TCSPC sample conditions compared to PAM sample
305 conditions. The decrease in both NPQ and NPQ τ was also stronger for longer HL periods as
306 well as later in the sequence (**Fig. 2**). The slope of the initial decrease in NPQ τ or NPQ during
307 HL was similar for all four sequences (**Supp. Fig. 12**) and is likely dictated by C_i availability
308 and its influence on the CCM kinetics. The magnitude of the decrease in NPQ and NPQ τ was
309 larger for longer light periods (**Supp. Fig. 12**), likely due to simultaneous activation of the
310 CCM that dissipates the proton gradient and the onset of slower forms of NPQ such as state
311 transitions. Conversely, the differences in the magnitude of the increase in NPQ and NPQ τ
312 during the dark periods could be due to differences in O_2 concentrations sensed by the cells in
313 both conditions, which is known to affect the extent and rate of state transition (Forti and
314 Caldiroli, 2005).

315

316 Although LHCSR3 plays the dominant role in photoprotection under constant and fluctuating
317 light conditions, we also observe a role for LHCSR1 in our measurements. While the
318 chlorophyll fluorescence dynamics of *lhcsr1* are similar to those of WT (**Fig. 3**), we observe a

319 ~20% reduction in overall NPQ in this mutant under fluctuating light conditions (**Fig. 6**). This
320 small amount of photoprotection afforded by LHCSR1 *in vivo* is consistent with previous *in*
321 *vitro* investigations (Dinc *et al.*, 2016; Nawrocki *et al.*, 2020) in which LHCSR1 has been
322 suggested to mediate energy transfer between LHCII and PSI (Kosuge *et al.*, 2018) and to
323 compensate for the absence of LHCSR3 (Girolomoni *et al.*, 2019). Interestingly, our results
324 suggest that different from the case for LHCSR3, the qE that is mediated by LHCSR1 is
325 largely frequency independent (**Supp. Fig. 8**). Both LHCSR1 and LHCSR3 are thought to
326 generate NPQ in response to (i) the proton gradient and (ii) carotenoid composition (Kondo *et*
327 *al.*, 2017; Troiano *et al.*, 2021), thus the frequency-dependent LHCSR3 and frequency-
328 independent LHCSR1 could differ in their pH or carotenoid dependency.

329

330 The relationship between NPQ capacities and growth have remained puzzling in
331 *Chlamydomonas*, because only some light fluctuation regimes have consistently been shown
332 to impair growth (Peers *et al.*, 2009; Truong, 2011; Cantrell and Peers, 2017; Roach, 2020).
333 Here we show that all mutants lacking LHCSRs showed impaired growth under rapid light
334 fluctuations (1-1 sequence), and that this impairment was lower under slower fluctuations (10-
335 10 sequence) and absent under an even slower 30-30 sequence or constant illumination (**Fig. 7**
336 and **Supp. Fig. 11**). There seems to be a good relationship between defect of NPQ and growth
337 deficiency under short time-scale fluctuations when considering *npq4lhcsr1* and *stt7npq4*
338 mutants (**Fig. 7**), clearly showing that LHCSR-dependent qE is critical for growth when light
339 fluctuates with short period of time. However, surprisingly, the growth defect of *lhcsr1*
340 seemed larger than *npq4* under the 1-1 sequence. This suggests that the growth capacity of
341 LHCSR mutants under light fluctuations may not depend only on the level of NPQ. Other
342 factors may include activation of compensatory mechanisms that enable photoprotection at
343 the expense of growth or the increased production of reactive oxygen species in *npq4* mutants
344 (Roach *et al.*, 2020; Barera *et al.*, 2021), which could be greater in the *lhcsr1* mutant. Note
345 here that in our conditions, due to slightly different genetic background between *stt7* and the
346 WT control, we cannot make a conclusion on the mechanism by which *stt7* grew better under
347 short timescale fluctuations (**Fig. 7**). The WT background may be particularly sensitive to fast
348 1-1 and 10-10 fluctuations; another possibility could be that qT is detrimental for growth
349 under medium to short time scale fluctuations.

350

351 Through 77K fluorescence emission spectra analysis, we have shown that the increasing
352 dissipation observed in the dark requires the STT7 kinase responsible for state transition (**Fig.**

353 **4 and Supp. Fig. 4).** This effect, already described in *Chlamydomonas* (Allorent et al., 2013)
354 and *Dunaliella salina* (Casper-Lindley and Björkman, 1996), greatly contributes to NPQ
355 during light fluctuations. In plants, the occurrence of state transitions and its involvement in
356 NPQ is thought to be minor (Allen, 1992; Minagawa, 2011) even if mutants of *Arabidopsis*
357 *thaliana* impaired in state transition (*stn7*) exhibit impaired growth under light fluctuations
358 (Bellafiore *et al.*, 2005). Interestingly, an increase of NPQ during the dark periods of
359 fluctuating light was recently reported in *npq4* leaves of *A. thaliana* (Steen et al., 2020) for
360 which about 53% of the WT NPQ τ remained in the mutant after 40 minutes of exposure to
361 light fluctuations despite the absence of the pH-sensing protein PsbS (**Supp. Fig. 13**).
362 However, it remains unclear as to how much of the dark quenching in plants originates from
363 qT as opposed to effects related to de-epoxidized xanthophyll pigments (Steen et al., 2020) or
364 LHC protein conformation and/or aggregation (Goral *et al.*, 2012). In the future, periodic
365 illumination experiments performed on *A. thaliana* mutants impaired in qE and/or qT could
366 clarify the relative importance of each mechanism and allow a comparison of the *in vivo*
367 functioning of NPQ in higher plants and green algae under light fluctuations.

368

369 Tuning the relaxation kinetics of NPQ in higher plants has been shown to improve crop plant
370 productivity (Kromdijk *et al.*, 2016), and recent modelling of the response of plant canopies to
371 natural light fluctuations has shown that there remains ample room for improving
372 photosynthetic efficiency under non-steady state conditions (Wang *et al.*, 2020). Similar
373 opportunities for improvement also exist for increasing biofuel production from microalgae
374 (Benedetti *et al.*, 2018; Perin and Jones, 2019; Vecchi *et al.*, 2020). In both cases, such
375 optimization will require detailed understanding of the dynamic activity of NPQ mechanisms.
376 Since the amount of LHCSR protein does not significantly differ between WT and *stt7* (**Supp.**
377 **Fig. 14**) and the sum of the NPQ contributions of each protein is greater than 100% (**Fig. 6**),
378 this suggests that there is an interaction between qE and qT under fluctuating light. The
379 possibility of an interaction between qE and qT has been previously suggested on the basis of
380 a kinetic analysis of qT in the presence and absence of LHCSR3 protein (*npq4* mutant)
381 (Roach and Na, 2017). Our findings are consistent with a partial overlap of the functions of
382 LHCSR3 and STT7 in both qE and qT. This partial overlap highlights the need for further
383 investigations of the interactions occurring between proteins that underlie the *in vivo* NPQ
384 response, not only in HL but also in dark, and more generally during light fluctuations. For
385 example, in microalgae, the quenching mediated by LHCSR3 both at PSII and PSI level
386 (Girolomoni et al., 2019) could be tuned by the movement of LHCSR3 from PSI to PSII

387 during state transitions (Allorent et al., 2013). LHCSR3 is known to associate with LHCII
388 trimers in the PSII supercomplex (Semchonok *et al.*, 2017); therefore, a similar LHCSR3-
389 LHCII interaction may also generate quenching in the trimers following the detachment of
390 LHCII from PSII. It should be noted that in the thylakoid membrane, LHCII can exist in a
391 range of different conformations and/or quenching states (Tian *et al.*, 2015; Kawakami *et al.*,
392 2019). At this point it is not possible to distinguish the relative contributions of different
393 forms of LHCII in individual snapshot measurements. The ensemble fluorescence lifetime
394 likely originates from some combination of at least three LHCII subpopulations:
395 unphosphorylated and bound to PSII (state 1) (Drop *et al.*, 2014) with an intermediate
396 fluorescence decay component, phosphorylated and unbound (free LHCII) (Iwai *et al.*, 2010b)
397 which has been previously assigned to a long fluorescence decay component (Ünlü et al.,
398 2014), and phosphorylated and bound to PSI (state 2) (Huang *et al.*, 2021) with a short
399 fluorescence decay component. In intact algal cells, the relative abundance of each form of
400 LHCII likely dynamically evolves throughout exposure to the fluctuating HL-dark sequences.
401 Further development of *in vivo* spectroscopic tools will be required to disentangle the
402 dynamics of LHCII conformations and correlate them with photoprotection. Overall, a deeper
403 understanding of the protein interactions underlying NPQ dynamics will be highly valuable in
404 finding new ways to improve plant and microalgal productivity.

405

406 **Conclusions**

407 LHCSR- and STT7-mediated nonphotochemical quenching processes (qE and qT) are known
408 to underly the photoprotective response of the microalgae *Chlamydomonas*. Here, we have
409 applied a new method to disentangle the involvement of qE and qT in real time by exposing
410 intact algal cells to repetitive cycles of high light and darkness alternating at different
411 frequencies. While both qE- and qT-type responses are present during all light fluctuations,
412 LHCSR-dependent qE plays a larger role in the beginning of light fluctuations and during the
413 HL periods. The contribution of STT7-dependent qT became more pronounced upon longer
414 exposure to fluctuating light and especially during the dark periods of light fluctuations. Over
415 the long term, rapid light fluctuations reduced the growth of mutants impaired in LHCSRs,
416 demonstrating the importance of LHCSR proteins during abrupt changes in light intensity.
417 Overall, our work suggests that a cooperativity between LHCSR proteins and STT7 may
418 constitute an important regulatory feature of the photoprotective response in *Chlamydomonas*.
419 These findings provide a valuable foundation for disentangling and modelling how the diverse
420 molecular mechanisms involved in plant and microalgal acclimation to light fluctuations

421 interact and enable robust photosynthesis in nature. We envision that further knowledge on
422 the response of photosynthetic mechanisms to various periods of light fluctuations will open
423 new avenues for building a strong understanding of how photosynthetic organisms respond to
424 complex light fluctuations.

425

426 **Accession numbers**

427 Genes studied in this article can be found on <https://phytozome.jgi.doe.gov/> under the loci
428 Cre08.g365900.t1.2 (LHCSR1), Cre08.g367500.t1.1 (LHCSR3.1), Cre08.g367400.t1.1
429 (LHCSR3.2), Cre02.g120250.t1.1 (STT7).

430 **List of abbreviations.** NPQ: non-photochemical quenching; qE: energy-dependent
431 quenching; qT: state transitions; qI: photoinhibition; qZ: zeaxanthin-dependent quenching;
432 PAM: pulse-amplitude modulation; TCSPC: time-correlated single photon counting; LHCSR:
433 light-harvesting stress related protein; STT7: serine/threonine-protein kinase; LHC: light-
434 harvesting complex; PS: photosystem; CCM: CO₂ concentration mechanism; C_i: inorganic
435 carbon (CO₂, HCO₃⁻, CO₃²⁻); HL: high light

436

437 **Materials and Methods**

438 **Strains and culture conditions**

439 *Chlamydomonas* mutants and their respective wild-type 4A- were previously described (*npq4*
440 (Peers et al., 2009), *lhcsr1* (Truong, 2011), *npq4lhcsr1* (Truong, 2011), *stt7* (Depege et al.,
441 2003), *stt7npq4* (Allorent et al., 2013)). All strains were grown photoautotrophically under
442 moderate light (50 μmol photons m⁻² s⁻¹) in minimal HS medium under air level of CO₂
443 (20°C). Except for the growth test, cell cultures (5-8 μg Chl mL⁻¹) were incubated overnight
444 at high light (400 μmol photons m⁻² s⁻¹), for maximizing expression of LHCSR proteins
445 (Tibiletti *et al.*, 2016). Prior to each measurement, cells were illuminated for at least 15 min
446 with low intensity far-red light (3in1LED panel with far-red LED; 3LH series, NK system,
447 Japan) to ensure a complete state 1 configuration (Bonaventura and Myers, 1969). All
448 replicates shown are biological replicates from independent cultures.

449

450 **Chlorophyll Fluorescence Measurements**

451 In this work, we employ two techniques to monitor the activation and deactivation of NPQ
452 throughout 40 minutes of exposure to repeated periods of high light and dark on the basis of
453 changes in Chl fluorescence emission (see **Fig. 1** for an illustration of the experimental
454 design). Time-resolved Chl fluorescence was measured via time-correlated single photon

455 counting (TCSPC) while Chl fluorescence yield was measured in parallel experiments using
456 pulse-amplitude modulation (PAM) fluorimetry. Although both methods can monitor NPQ,
457 the fluorescence lifetime is not susceptible to a range of non-quenching processes that can
458 impact the fluorescence yield (such as chromophore bleaching, changes in chlorophyll
459 concentration, chloroplast movement, or enhanced light scattering (Zaks et al., 2013; Sylak-
460 Glassman *et al.*, 2016). Therefore, fluorescence lifetime measurements provide insight into
461 processes that directly quench chlorophyll fluorescence.

462 **A) TCSPC measurement and fitting**

463 The average chlorophyll fluorescence lifetime was measured by time-correlated single
464 photon counting (TCSPC), as previously described (Sylak-Glassman et al., 2016; Steen et al.,
465 2020). A diode laser (Coherent Verdi G10, 532 nm) pumped a Ti:Sapphire oscillator
466 (Coherent Mira 900f, 808 nm, 76 MHz) and the output was subsequently frequency doubled
467 using a β -barium borate crystal to obtain 404 nm light. These pulses were used for excitation
468 of the sample with a power of 1.7 mW (20 pJ/pulse) and Chl fluorescence emission at 680 nm
469 was detected via an MCP-PMT (Hamamatsu R3809U). A custom-built LABVIEW software
470 was used to synchronize three shutters located in the laser path, actinic light path, and the path
471 between the sample and detector. Every 15 sec, a fluorescence lifetime snapshot measurement
472 was acquired by exposing the cells to the saturating laser (404 nm) for 1 second and detecting
473 the emission. Fluorescence lifetime snapshots were measured by TCSPC using a Becker-
474 Hickl SPC-850 data acquisition card and SPCM software. In between the snapshot
475 measurements, high-light illumination of the cells was achieved by exposing the cuvette to
476 white light set to an intensity of $620 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Leica KL1500 LCD, peak 648 nm,
477 FWHM 220 nm). The sample concentration was adjusted to $\sim 80 \mu\text{g Chl mL}^{-1}$ for TCSPC
478 measurements. To control the gas composition of the culture and prevent cells from settling to
479 the bottom of the cuvette, the sample was bubbled with air (ambient CO_2 concentrations) at a
480 rate of $2\text{-}7 \text{ mL min}^{-1}$ throughout the entire 40 min experiment duration, although note that
481 such bubbling increased the noise of the measurements.

482 For each fluorescence decay measurement, to ensure that PSII reaction centers were
483 closed, we selected the 0.2 s step with the longest lifetime from the overall 1 s snapshot
484 measurement duration (Sylak-Glassman et al., 2016). This longest step was then fit to a bi-
485 exponential decay (Picoquant, Fluofit Pro-4.6) and the average amplitude-weighted
486 fluorescence lifetime (τ_{avg}) was calculated for each snapshot measurement. The NPQ τ
487 parameter is derived from the fluorescence lifetime snapshot measurements and is defined
488 analogously to NPQ (Sylak-Glassman et al., 2014; Sylak-Glassman et al., 2016): $\text{NPQ}\tau(t) =$

489 $\frac{\tau_{avg}(0) - \tau_{avg}(t)}{\tau_{avg}(t)}$. The value of NPQ τ represents the magnitude of the quenching response based
490 on the change in the average fluorescence lifetimes between time $t=0$ (after far-red
491 acclimation but before HL exposure) and any other time t during the 40 min snapshot
492 trajectory. Therefore, using NPQ τ removes confounding effects arising from any differences
493 in the average chlorophyll excited state lifetime of the different strains following far-red
494 acclimation. For all TCSPC measurements, each biological replicate represents the average of
495 three technical replicates measured on the same day.

496

497 ***B) PAM measurements***

498 Chlorophyll fluorescence yield was measured using a pulsed-amplitude modulation
499 (PAM) fluorimeter (Dual-PAM 100, Walz GmbH, Effeltrich, Germany) with the red
500 measuring head. Red saturating flashes (8,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 600 ms, 620 nm) were
501 delivered to measure F_M (maximal fluorescence yield in dark-acclimated samples) and then
502 every 15 s or 30 s to measure F_M' under actinic light exposure or dark phase respectively.
503 Actinic light illumination (620 nm) was set to 620 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Fluorescence
504 emission was detected using a long-pass filter ($>700 \text{ nm}$). NPQ was calculated as $(F_M -$
505 $F_M')/F_M'$. The Chl concentration was $\sim 5\text{-}8 \mu\text{g Chl mL}^{-1}$ and as for TCSPC, all PAM
506 measurements and the sample was bubbled with air at a flux of 2-7 mL min^{-1} for proper
507 control of the gas concentrations of the sample throughout the entire 40 min experiment
508 duration, note that such bubbling increased the noise of the measurements (but to a lesser
509 extent than for TCSPC).

510

511 ***C) Quantifying the contributions of LHCSR1, LHCSR3, and STT7 to NPQ***

512 To assess the relative contributions of LHCSR1, LHCSR3, and STT7 to overall NPQ,
513 we analyzed the NPQ (PAM) and NPQ τ (TCSPC) trajectories for WT and each mutant. The
514 relative contribution of each protein was determined as the percent change in the integrated
515 snapshot trajectory of NPQ or NPQ τ for each mutant relative to the control WT strain. As the
516 contribution of each actor was found to be overall independent of HL-dark fluctuation
517 frequencies in the range of 1 min^{-1} to 0.1 min^{-1} . (**Supp. Fig. 8**), the average contribution of
518 each protein under the four light fluctuating sequences for both PAM and TCSPC was
519 considered. Additionally, to characterize the involvement in activation or deactivation of
520 NPQ, the quenching trajectories were integrated solely under HL or dark periods, respectively
521 (**Supp. Fig. 9**). The contributions of each protein to the early vs. late responses were further

522 assessed by integrating from 0-20 min and 20-40 min, respectively (**Supp. Fig. 10**). These
523 results are summarized in **Fig. 6**.

524

525 **77K Chlorophyll fluorescence emission**

526 Chlorophyll fluorescence emission spectra of *Chlamydomonas* cells at 77 K were obtained by
527 freezing whole cells (~5-8 $\mu\text{g Chl mL}^{-1}$ final concentration) in liquid nitrogen. The emission
528 spectrum was then measured between 600 and 800 nm (435 nm excitation wavelength, RF-
529 5300PC spectrophotometer, Shimadzu).

530

531 **Growth tests**

532 The different *Chlamydomonas* strains were cultivated photoautotrophically under moderate
533 light (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in minimal medium under air level of CO_2 (20°C). Cells were
534 harvested during exponential growth and resuspended in fresh minimal medium to 0.1, 0.5, or
535 2 $\mu\text{g Chl mL}^{-1}$. Eight-microliter drops were spotted on minimal medium plates at pH=7.2 and
536 exposed to the various light conditions. Homogeneous light was supplied by LED panels.
537 Temperature was maintained at 25°C at the level of plates.

538

539

540 **Acknowledgments:**

541 We thank Dr. Setsuko Wakao for assistance in growing cells, Jacob Irby for performing
542 immunodetection, and Dr. Guillaume Allorent and Dr. Giovanni Finazzi for providing the
543 *npq4stt7* strain. This work was supported by the U.S. Department of Energy, Office of
544 Science, Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division
545 under the field work proposal 449B. K.K.N. is an investigator of the Howard Hughes Medical
546 Institute.

547

548 **Competing interests:** The authors declare that they have no competing interest.

549

550 **Data and materials availability:** All data needed to evaluate the conclusions in the paper are
551 present in the paper and/or the Supplementary Materials.

552

553 **List of Figures, Tables, and Supporting Material:**

554

555 **Figure 1.** Experimental design for Chl *a* fluorescence measurements throughout exposure of
556 *Chlamydomonas* cells to fluctuating light with various periods of HL-dark exposure. (**A, B**)
557 Representation of the HL-dark cycles used for the 40 minutes of light fluctuation and their
558 corresponding period, frequency, and name used throughout the main text. NPQ was
559 measured using Pulsed Amplitude Modulation (PAM, **C**) and Time-Correlated Single Photon
560 Counting (TCSPC, **D**). (**C**) Characteristics of the PAM measurement and representative data
561 of fluorescence yield in WT cells. (**D**) Characteristics of the TCSPC apparatus. Shown are
562 two decays representative of two snapshots taken in a quenched and unquenched state.

563

564 **Figure 2.** Quenching trajectories during light fluctuations in *npq4lhcsr1* and its control strain.
565 The response of NPQ and NPQ τ (upper and lower panel respectively) were measured in
566 *npq4lhcsr1* mutant and its control strain (red and blue curves respectively) during 40 minutes
567 of light fluctuations with periods of 1, 2, 4 and 10 minutes (**A, B, C** and **D** respectively) as
568 described in **Fig. 1**. Shown are average of three biological replicates. For TCSPC data, each
569 biological replicate was averaged from three technical replicates. The fluorescence lifetime
570 values used to calculate NPQ τ are shown in the Supplemental.

571

572 **Figure 3.** Quenching trajectories during light fluctuations in *lhcsr1* and *npq4*. The response of
573 NPQ and NPQ τ (upper and lower panel respectively) were measured in *lhcsr1* and *npq4*
574 (purple and orange curves respectively) during 40 minutes of light fluctuations with periods of
575 1, 2, 4 and 10 minutes (**A, B, C** and **D** respectively) as described in **Fig. 1**. Shown are average
576 of three biological replicates. For TCSPC data, each biological replicate was averaged from
577 three technical replicates. The fluorescence lifetime values used to calculate NPQ τ are shown
578 in the Supplemental.

579

580 **Figure 4.** 77K chlorophyll fluorescence emission spectra during the first high light-dark cycle
581 of light fluctuations. Cells were placed in a TCSPC cuvette as described in **Fig. 1** and both
582 fluorescence lifetime snapshots and 77K chlorophyll fluorescence emission spectra were
583 taken through 10 minutes of high light and 10 minutes darkness. (**A**) Fluorescence lifetime
584 trajectory of *npq4lhcsr1* mutant (red dots) and its control strain (WT, blue dots). On the
585 graph, dashed vertical lines depict the timepoints at which samples were taken for 77K
586 fluorescence spectra measurement. (**B, C**) 77K fluorescence emission spectra of samples
587 taken in **A** on the control strain (WT, **B**) and *npq4lhcsr1* mutant (**C**). Spectra were taken at 0,
588 10, and 20 min timepoints (blue, orange and grey spectra respectively). Shown are
589 representative spectra. Three independent biological replicate spectra for WT and *npq4lhcsr1*
590 are shown in **Supp. Fig. 3**. 77K spectra for the *stt7* and *stt7npq4* strains are shown in **Supp.**
591 **Fig. 4**.

592

593 **Figure 5.** Quenching trajectories during light fluctuations in *stt7* and *stt7npq4*. The response
594 of NPQ and NPQ τ (upper and lower panel respectively) were measured in *stt7* and *stt7npq4*
595 (green and magenta curves respectively) during 40 minutes of light fluctuations with periods
596 of 1, 2, 4 and 10 minutes (**A, B, C** and **D** respectively) as described in **Fig. 1**. Shown are
597 average of three biological replicates. For TCSPC data, each biological replicate was
598 averaged from three technical replicates. The fluorescence lifetime values used to calculate
599 NPQ τ are shown in the Supplemental.

600

601 **Figure 6.** Quantification of the contribution of LHCSRs and STT7 to wild-type NPQ under
602 fluctuating light. (**A**) Example of quantification of the relative NPQ mediated by LHCSRs.
603 The area under the NPQ curve of *npq4lhcsr1* mutant (red) was subtracted from that of the
604 control strain (blue) and expressed relative to the area of NPQ of the control strain. (**B, C**)

605 Overall contribution of LHCSRs (red), LHCSR3 (orange), LHCSR1 (purple) and STT7
606 (green) averaged over all 40 minutes (**B**) or specific periods of the light fluctuations (**C**). Each
607 donut portrays the amount of wild-type NPQ that is lost in each mutant impaired in the
608 accumulation of the given protein. Given that the contribution of each protein was largely
609 independent of HL/dark period (**Supp. Fig. 6**), shown are the average of all 4 light fluctuation
610 sequences. Distribution of individual replicates and estimates of error are presented in **Supp.**
611 **Fig. 8-10**.

612

613 **Table 1.** Average contribution of each protein to overall wild-type NPQ for each light
614 fluctuation sequence. Shown is the average value (n=6, evaluated from 3 TCSPC and 3 PAM
615 replicates) and standard deviation of all individual replicates. The contributions of LHCSR3
616 (orange) and LHCSR1 (purple) were determined from the single mutants *npq4* and *lhcsr1*.
617 The contribution of LHCSRs overall (red) was evaluated from the *npq4lhcsr1* mutant. The
618 contribution of qT was assessed from the *stt7* mutant. Each error in the right column
619 represents the standard deviation of each protein's contribution across the 4 light fluctuation
620 sequences. For simplicity, only the average values (shown in the right column) were used to
621 generate **Figure 6** in the main text. Full distributions of the individual TCSPC and PAM data
622 points are shown in **Supp. Fig. 8**. [supports Fig. 6B]

623

624 **Figure 7.** Growth of mutants impaired in qE and/or qT under various periods of dark/light
625 cycles. *lhcsr1*, *npq4*, *npq4lhcsr1*, *stt7* and *stt7npq4* mutants and their control strain (WT) were
626 diluted and spotted at different chlorophyll concentration and grown on plates under
627 dark/light cycles with a period of 30 (30-30, upper panel), 10 (10-10, middle panel) or 1
628 minute (1-1, lower panel). Each row represents a different chlorophyll concentration. Shown
629 are representative spots of three biological replicates. Growth under constant low light or high
630 light are shown in Supporting Figure 7.

631

632 **Supporting Materials:**

633 Quenching trajectories with error (standard deviation) for WT and *npq4lhcsr1* (SI Fig 1)

634 Quenching trajectories with error (standard deviation) for *lhcsr1* and *npq4* (SI Fig 2)

635 77K emission spectra PAM replicates for WT and *npq4lhcsr1* (SI Fig 3)

636 77K emission spectra for *stt7* and *stt7npq4* (SI Fig 4)

637 Quenching trajectories with error (standard deviation) for *stt7* and *stt7npq4* (SI Fig 5)

638 Maximum quenching envelopes for WT and *stt7* (SI Fig 6).

639 Kinetics of qT in WT and *npq4lhcsr1* (SI Fig 7).

640 Quantification of protein contributions: distributions, averages, errors (SI Fig 8-10).

641 Growth of cells under constant LL or HL (SI Fig 11).

642 Kinetics of CCM-related decrease in WT NPQ during HL (SI Fig 12)

643 Comparison of integration results for WT and pH-sensing mutant in *Chlamydomonas* and

644 *Arabidopsis* (SI Fig 13)

645 Immunodetection of LHCSR proteins (SI Fig 14)

646

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648

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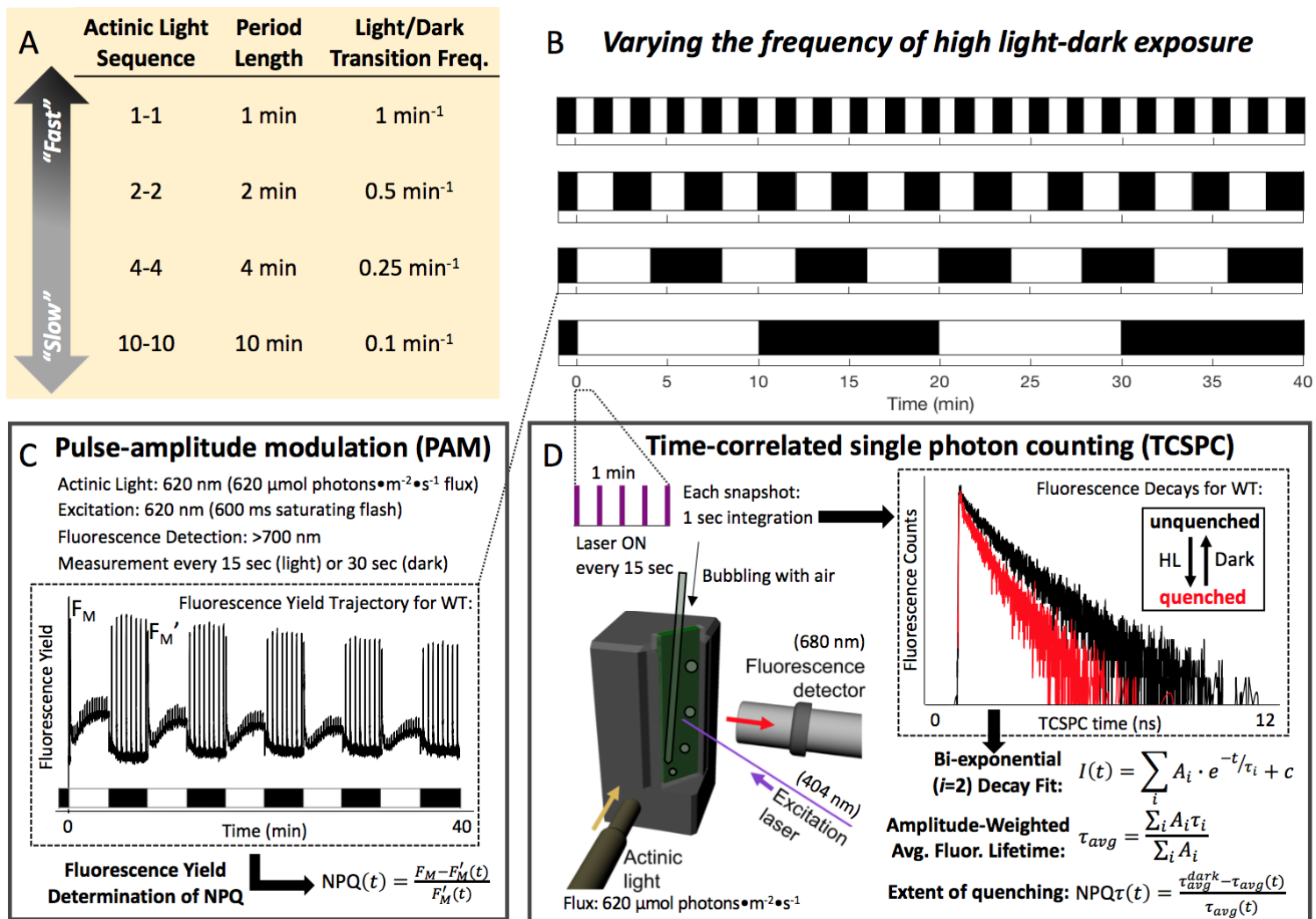


Figure 1. Experimental design for Chl *a* fluorescence measurements throughout exposure of *Chlamydomonas* cells to fluctuating light with various periods of HL-dark exposure. **(A, B)** Representation of the HL-dark cycles used for the 40 minutes of light fluctuation and their corresponding period, frequency, and name used throughout the main text. NPQ was measured using Pulsed Amplitude Modulation (PAM, **C**) and Time-Correlated Single Photon Counting (TCSPC, **D**). **(C)** Characteristics of the PAM measurement and representative data of fluorescence yield in WT cells. **(D)** Characteristics of the TCSPC apparatus. Shown are two decays representative of two snapshots taken in a quenched and unquenched state.

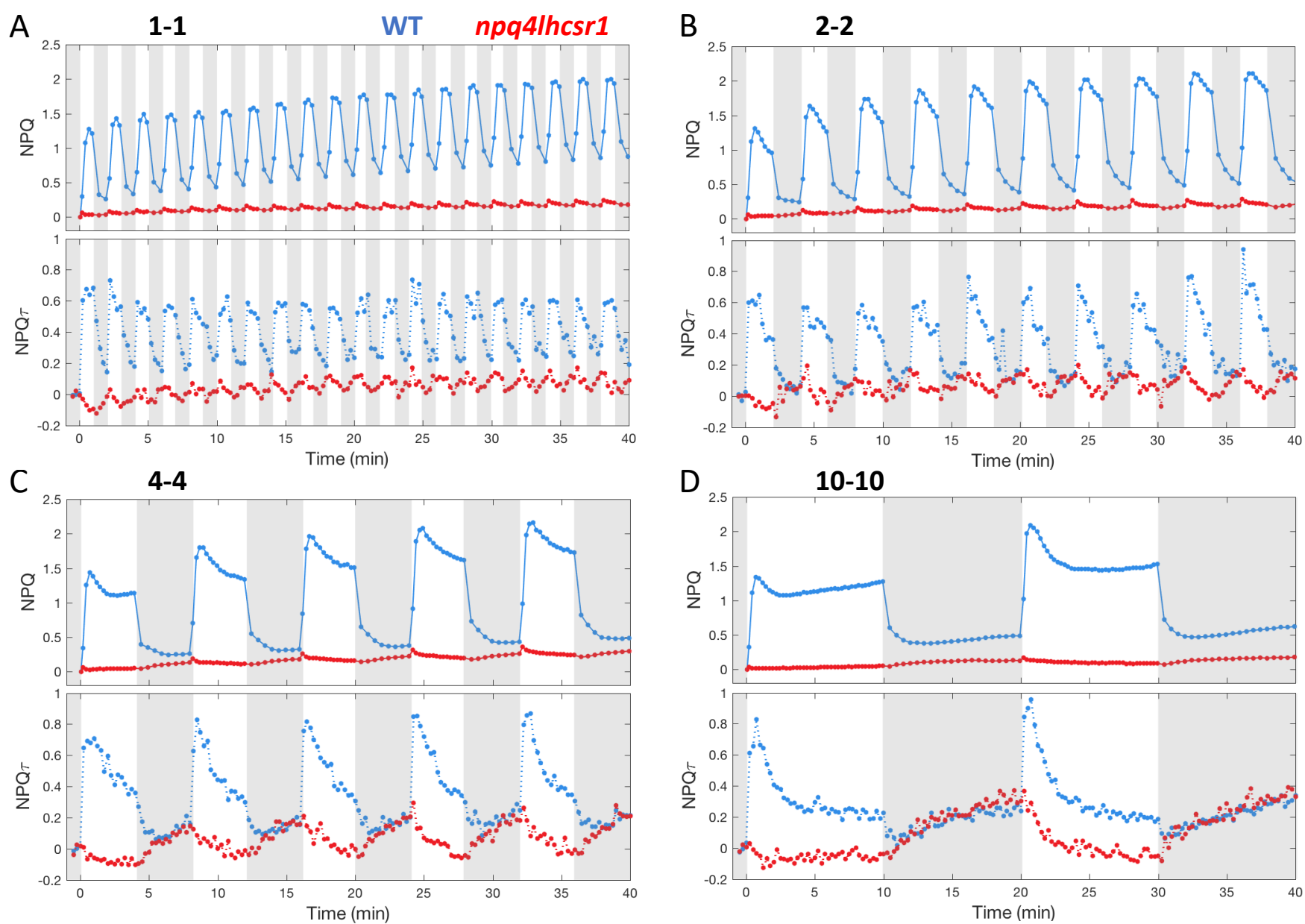


Figure 2. Quenching trajectories during light fluctuations in *npq4lhcsr1* and its control strain. The response of NPQ and NPQ τ (upper and lower panel respectively) were measured in *npq4lhcsr1* mutant and its control strain (red and blue curves respectively) during 40 minutes of light fluctuations with periods of 1, 2, 4 and 10 minutes (A, B, C and D respectively) as described in Fig. 1. Shown are average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ τ are shown in the Supplemental.

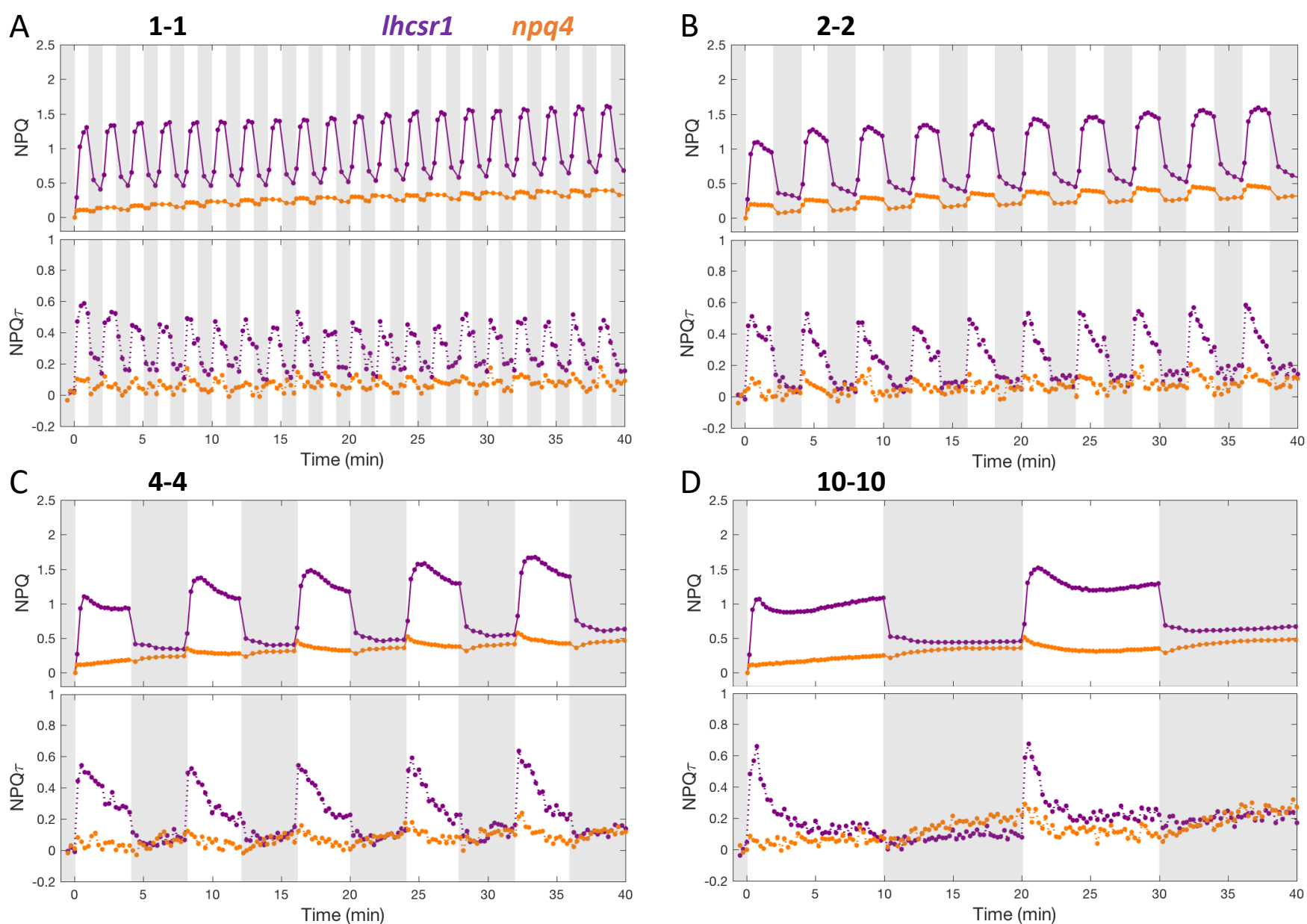


Figure 3. Quenching trajectories during light fluctuations in *lhcsr1* and *npq4*. The response of NPQ and NPQ τ (upper and lower panel respectively) were measured in *lhcsr1* and *npq4* (purple and orange curves respectively) during 40 minutes of light fluctuations with periods of 1, 2, 4 and 10 minutes (A, B, C and D respectively) as described in Fig. 1. Shown are average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ τ are shown in the Supplemental.

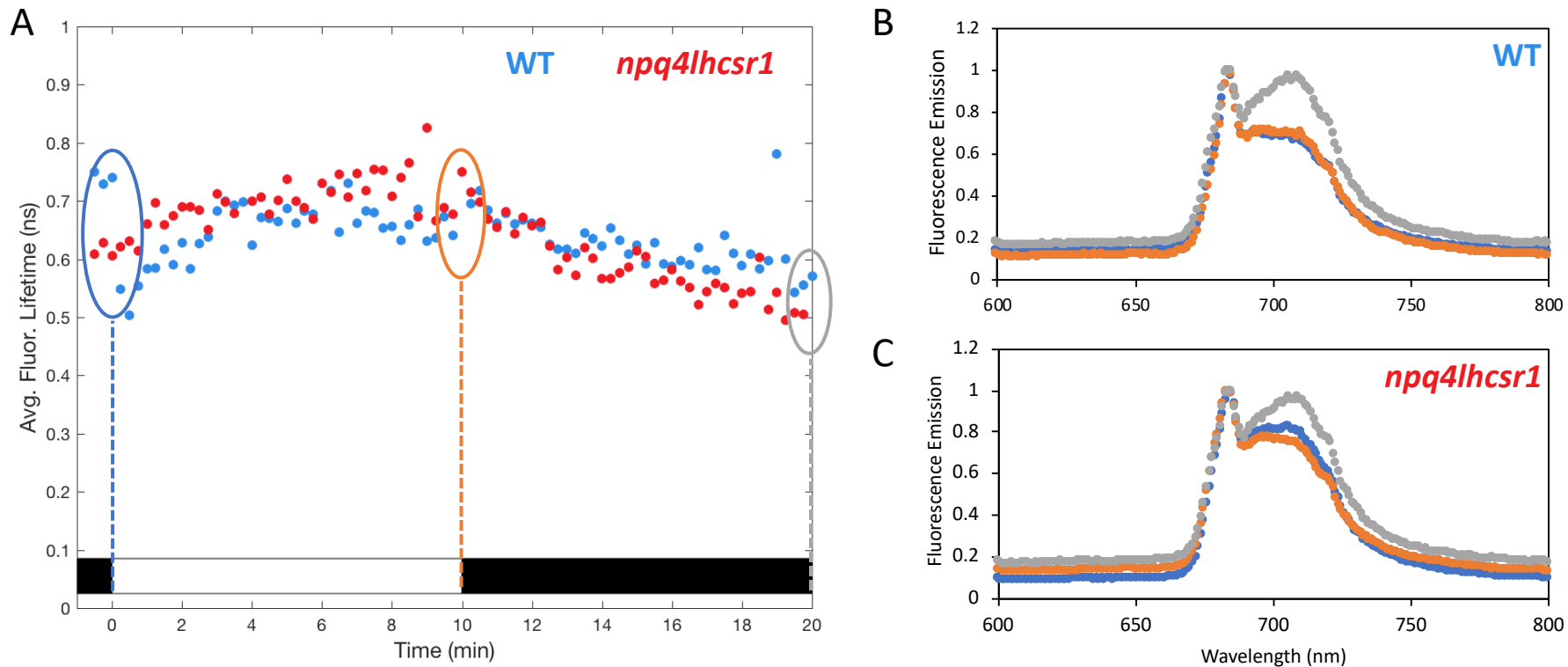


Figure 4. 77K Chlorophyll fluorescence emission spectra during the first high light-dark cycle of light fluctuations. Cells were placed in a TCSPC cuvette as described in **Fig. 1** and both fluorescence lifetime snapshots and 77K chlorophyll fluorescence emission spectra were taken through 10 minutes of high light and 10 minutes darkness. **(A)** Fluorescence lifetime trajectory of *npq4lhcsr1* mutant (red dots) and its control strain (WT, blue dots). On the graph, dashed vertical lines depict the timepoints at which samples were taken for 77K fluorescence spectra measurement. **(B, C)** 77K fluorescence emission spectra of samples taken in **A** on the control strain (WT, **B**) and *npq4lhcsr1* mutant (**C**). Spectra were taken at 0, 10, and 20 min timepoints (blue, orange and grey spectra respectively). Shown are representative spectra. Three independent biological replicate spectra for WT and *npq4lhcsr1* are shown in **Supp. Fig. 3**. 77K spectra for the *stt7* and *stt7npq4* strains are shown in **Supp. Fig. 4**.

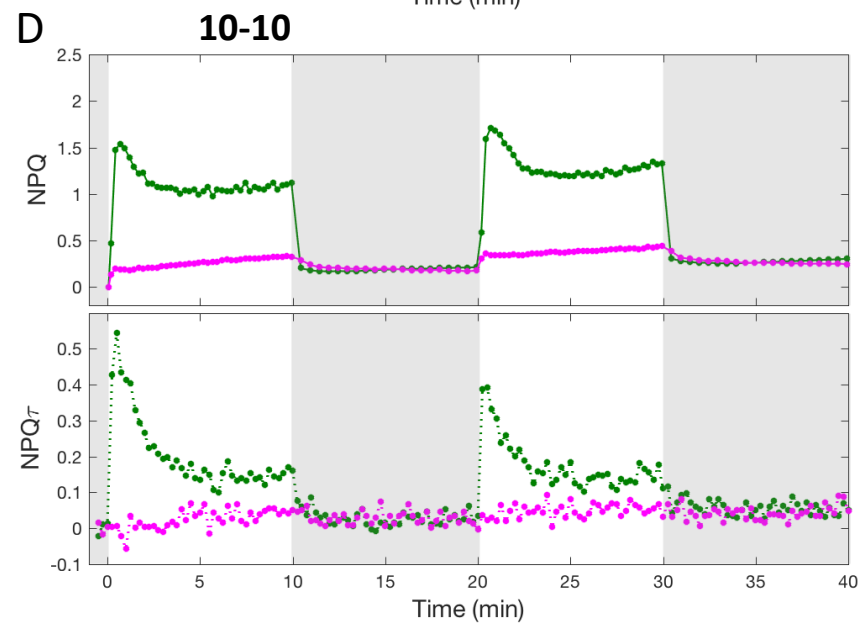
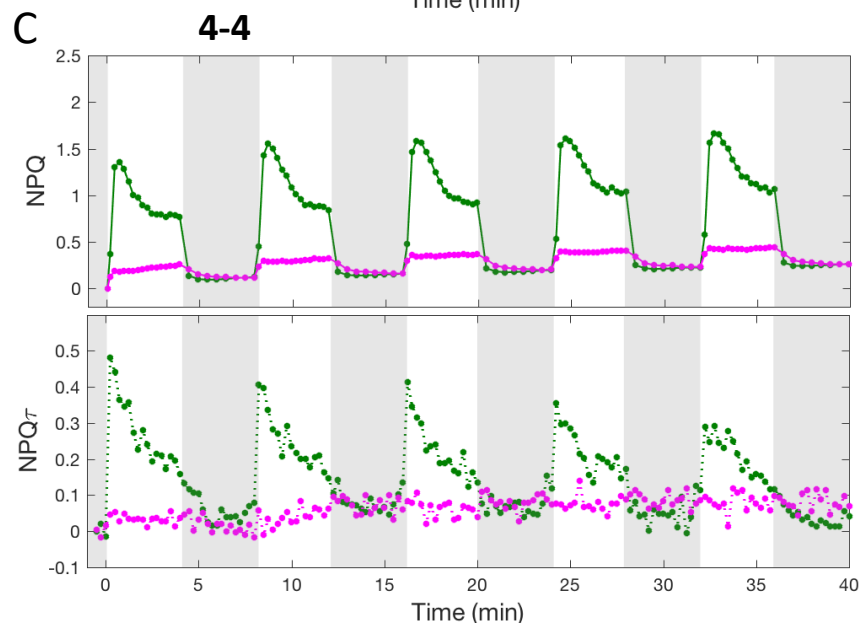
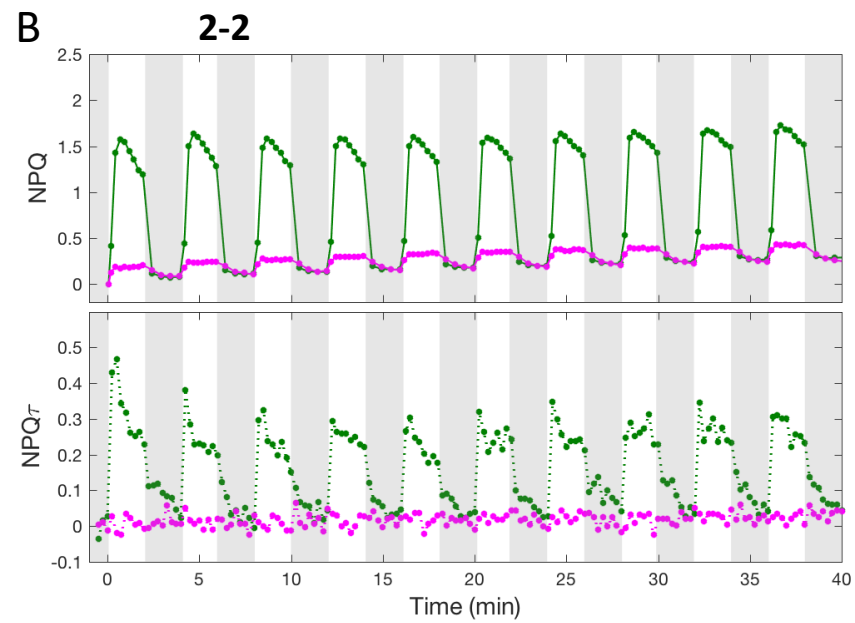
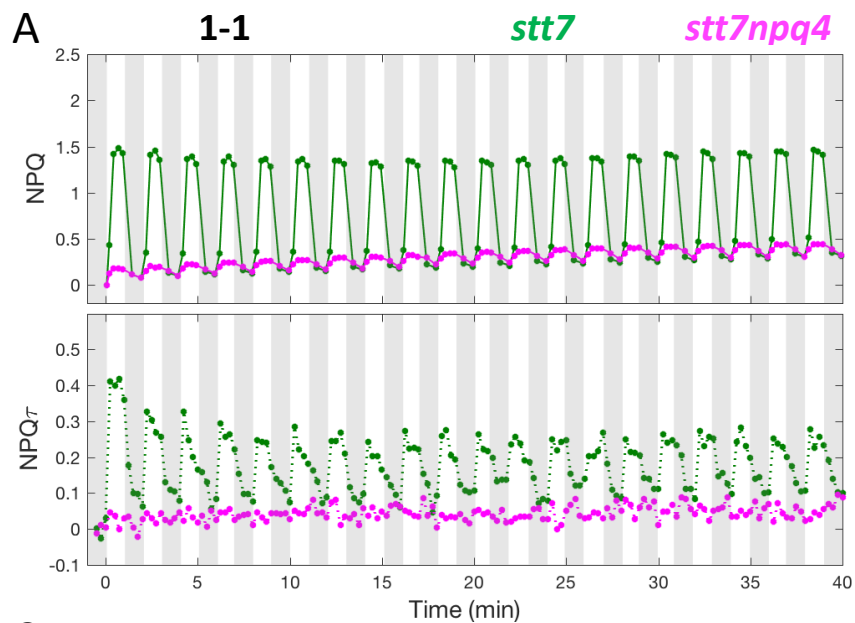


Figure 5. Quenching trajectories during light fluctuations in *stt7* and *stt7npq4*. The response of NPQ and NPQ τ (upper and lower panel respectively) were measured in *stt7* and *stt7npq4* (green and magenta curves respectively) during 40 minutes of light fluctuations with periods of 1, 2, 4 and 10 minutes (**A**, **B**, **C** and **D** respectively) as described in **Fig. 1**. Shown are average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ τ are shown in the Supplemental.

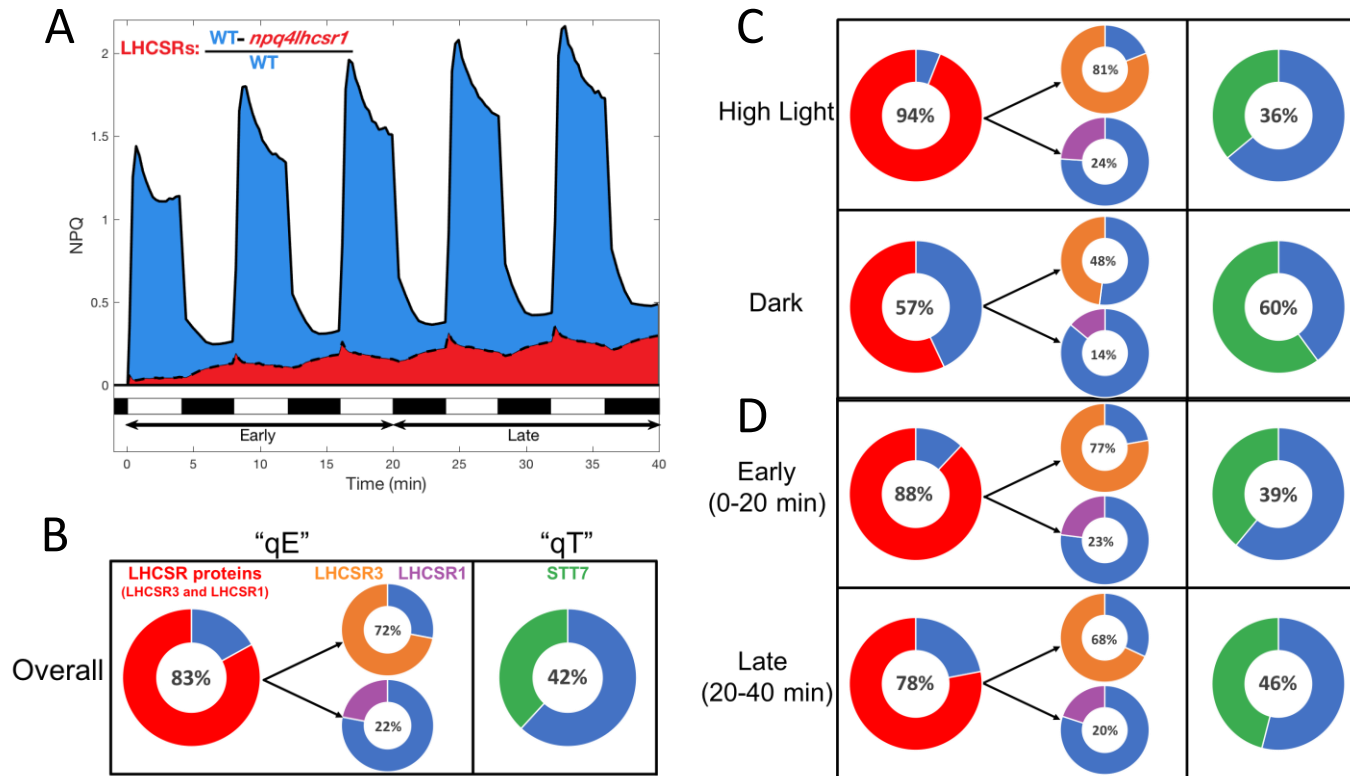


Figure 6. Quantification of the contribution of LHC SRs and STT7 to wild-type NPQ under fluctuating light. **(A)** Example of quantification of the relative NPQ mediated by LHC SR proteins. The area under the NPQ curve of *npq4lhcsr1* mutant (red) was subtracted from that of the control strain (blue) and expressed relative to the area of NPQ of the control strain. **(B)** Overall contribution of LHC SRs (red), LHC SR3 (orange), LHC SR1 (purple) and STT7 (green) averaged over all 40 minutes, **(C)** during HL and dark portions of the light fluctuations, or **(D)** during early (0-20 min) and late (20-40 min) portions of the experiment. Each donut portrays the amount of wild-type NPQ that is lost in each mutant impaired in the accumulation of the given protein. Given that the contribution of each protein was largely independent of HL/dark period (**Supp. Fig. 8**), shown here are the average of all 4 light fluctuation sequences. Distribution of individual replicates and estimates of error are presented in **Supp. Fig. 8-10**.

Table 1. Average contribution of each protein to overall wild-type NPQ for each light fluctuation sequence. Shown is the average value (n=6, evaluated from 3 TCSPC and 3 PAM replicates) and standard deviation of all individual replicates. The contributions of LHCSR3 (orange) and LHCSR1 (purple) were determined from the single mutants *npq4* and *lhcsr1*. The contribution of LHCSRs overall (red) was evaluated from the *npq4lhcsr1* mutant. The contribution of qT was assessed from the *stt7* mutant. Each error in the right column represents the standard deviation of each protein’s contribution across the 4 light fluctuation sequences. For simplicity, only the average values (shown in the right column) were used to generate **Figure 6** in the main text. Full distributions of the individual TCSPC and PAM data points are shown in **Supp. Fig. 8**. [supports Fig. 6B]

Overall Contributions	1-1	2-2	4-4	10-10	AVERAGE
LHCSR_s	89 ± 7%	84 ± 7%	83 ± 13%	77 ± 24%	83 ± 5%
LHCSR1	22 ± 12%	22 ± 10%	24 ± 16%	19 ± 30%	22 ± 2%
LHCSR3	81 ± 5%	78 ± 5%	73 ± 8%	58 ± 15%	72 ± 10%
STT7	46 ± 12%	38 ± 16%	46 ± 13%	39 ± 22%	42 ± 4%

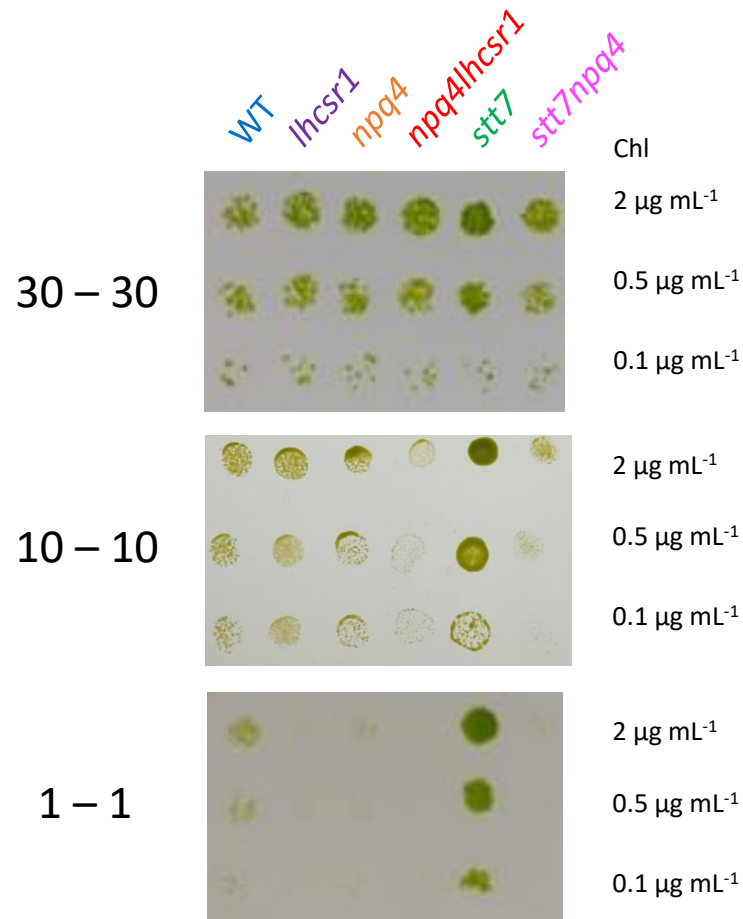


Figure 7. Growth of mutants impaired in qE and/or qT under various periods of dark/light cycles. *lhcsr1*, *npq4*, *npq4lhcsr1*, *stt7* and *stt7npq4* mutants and their control strain (WT) were diluted and spotted at different chlorophyll concentration and grown on plates under dark/light cycles with a period of 30 (30-30, upper panel), 10 (10-10, middle panel) or 1 minute (1-1, lower panel). Each row represents a different chlorophyll concentration. Shown are representative spots of three biological replicates. Growth under constant low light or high light are shown in **Supp. Fig. 11**.