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# 1 **Standard units for ElectroChromic Shift (ECS) measurements in plant biology**

2

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4

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8

## 9 **Highlight**

10 ElectroChromic Shift can be used to measure the light-response curves of electron transport  
11 rate, the fraction of open photosystems and a transmission coefficient for the photosynthetic  
12 chain.

13

## 14 **Abstract**

15 The absorbance shift of pigments is proportional to the membrane potential ( $\Delta\psi$ ) in plants,  
16 green algae, and many photosynthetic bacteria. It is currently denoted as ElectroChromic Shift  
17 (ECS) at 515-520 nm for plant carotenoids. It is increasingly being used for phenotyping  
18 plants for traits related to photosynthesis or chloroplast metabolism because it is a non-  
19 invasive technique and also because more instruments are now commercially available from  
20 various manufacturers. The ECS technique is currently used to monitor the post-illumination  
21 decay of the proton-motive force (*pmf*), but it has a yet more general use for quantitative  
22 studies on photosynthetic energy transduction. Here we briefly summarize the basic  
23 knowledge on ECS, emphasize the full potential of this technique, and propose a quantitative  
24 analysis of the photosynthetic performance with the definition of a transmission coefficient  
25 for electrons along the photosynthetic chain.

26

27

## 28 **Keywords**

29 Photosynthesis, chloroplast, thylakoid, cyclic electron flow, ATP-synthase

30

## 31 **Abbreviations**

32 ECS, ElectroChromic Shift

33 *pmf*, proton-motive force

34  $\Delta\psi$ , membrane potential

35  $I$ , initial electron flow rate  
36  $J$ , steady-state electron flow rate  
37 PS<sub>o</sub>, open photosystems  
38

### 39 **Introduction**

40 ECS signal was first reported as P515 (Duysens, 1954), an absorbance increase ( $\Delta A$ ) peaking  
41 around 515 nm. This signal was attributed to a change in absorption of carotenoids (Witt,  
42 1979), proportional to the membrane potential ( $\Delta\psi$ ). This signal was then used for the study  
43 of proton flow through ATPase (Junge, 1970) and the "slow" electrogenic phase of *cyt b<sub>6</sub>f*  
44 complex (Joliot and Delosme, 1974). More recently, the ECS decay rate after a continuous  
45 illumination was related to photosynthetic electron flow, allowing this technique to measure a  
46 flux as for any other reaction intermediate in the photosynthetic chain (Sacksteder and  
47 Kramer, 2000). This concept deserves explanation: if the ECS signal is constant over time, as  
48 observed at steady-state under continuous illumination, then the reactions building the proton-  
49 motive force (light-induced electron and proton transport) are exactly compensated by those  
50 consuming the proton-motive force (by ATPase in the dark), so that the post-illumination  
51 decay rate of ECS (membrane conductivity  $g_{H^+}$ , in  $s^{-1}$ ) is exactly equal to the electron transfer  
52 rate through the photosystems. This contribution had a very broad impact on the  
53 photosynthesis and chloroplast research community, it allowed for semi-quantitative studies  
54 of energy-dependent non-photochemical chlorophyll fluorescence quenching (qE) in relation  
55 to electron and proton flow (Kanazawa and Kramer, 2002), of cyclic and linear electron flows  
56 by comparing WT and *pgr5* plants (Avenson et al., 2005), of the parsing of the *pmf* between  
57  $\Delta\psi$  and  $\Delta pH$  (Cruz et al., 2001) and the recent identification of the thylakoid proton antiporter  
58 KEA3 (Kunz et al., 2014).

59  
60 The rate of a reaction (in  $s^{-1}$ ) defines enzyme kinetics. Yet, for the characterization of an  
61 integrated pathway like photosynthesis, the flux (in number of particles, per unit time per unit  
62 area) is a much more relevant parameter. Kramer and co-workers showed that the amplitude  
63 of the ECS decay is proportional to the proton motive force (*pmf*). Therefore, the efflux of  
64 protons through ATPase ( $v_{H^+}$ ) can be related to its conductivity  $g_{H^+}$  as follows:  
65  $v_{H^+} \sim pmf \times g_{H^+}$  (Avenson et al., 2004). The units for this proton efflux are not always  
66 specified (often in arbitrary units) but they should be  $\mu mol_{H^+} m^{-2} s^{-1}$ , like the electron  
67 transport rate (ETR) is expressed in  $\mu mol e^- m^{-2} s^{-1}$ . An uncertainty lies with these units as  
68 they depend on the amount of photons absorbed (and electrons or protons transferred) per leaf

69 area. Therefore we would like to follow up on an idea that was proposed previously (Joliot  
70 and Joliot, 2002): ECS can be calibrated on an internal standard to determine the number of  
71 electrons transferred per photosystem per unit time ( $e^- PS^{-1} s^{-1}$ ). Here the unit area is the  
72 functional unit of photosynthesis, the photosystems themselves.

73

## 74 **Materials and Methods**

75

76 *Chlorella sorokiniana* WT, and chloroplast ATPase-deficient mutant SL8 (Rappaport et al.,  
77 1999) were heterotrophically grown at 20°C, in continuous light ( $20 \mu\text{mol}_{\text{photons}} \text{m}^{-2} \text{s}^{-1}$ ), in  
78 Tris-acetate-phosphate medium. Prior to each experiment, cells were concentrated and  
79 resuspended in 10% Ficoll to a concentration of  $\sim 15 \mu\text{g}_{\text{Chl}} \text{mL}^{-1}$ , then adapted for >15 min in  
80 Erlenmeyer flasks stirred at 150 rpm prior to measurements. 3-(3,4-dichlorophenyl)-1,1-  
81 dimethyl-urea (DCMU) was used at a final concentration of 10  $\mu\text{M}$ , hydroxylamine at 10  
82 mM.

83

84 *Absorption spectroscopy* - The ElectroChromic Shift (ECS) of carotenoids was measured as  
85 an absorbance changes ( $\Delta A$ ) with a Joliot-type spectrophotometer using a Xenon flashlamp  
86 fitted to a Jobin-Yvon HL300 f/2 monochromator (Joliot et al., 1980) or to a white LED fitted  
87 to a 520 nm band pass dielectric filter (JTS-10, Biologic). Similar measurements can be done  
88 with Walz Dual-Pam 100 or MultispeQ. The detecting photodiodes were protected by a  
89 combination of BG39 (Schott) and CVI low-pass dielectric filters (600 nm). Action spectra  
90 were obtained with continuous light provided with a SPEX illuminator as excitation light (a  
91 Xenon arc lamp fitted to a Jobin Yvon 1681 Minimate f/4 monochromator) and computer-  
92 controlled shutter (Uniblitz).

93 The JTS-10 is supplied with a detection filter centered at 546 nm that can be used for  
94 deconvolution of cytochrome *f* (554 nm) or *b* (563 nm) signals. We find that, while it is  
95 necessary to correct for cytochrome kinetics, it was not for ECS in the present study where  
96 illuminations were rather short ( $< 10$  s). For longer illuminations, inducing de-epoxidation of  
97 violaxanthin to zeaxanthin (Illoiaia et al., 2011), thylakoid membrane swelling and scattering  
98 changes or chloroplast migration in plant leaves, it would become necessary to extract pure  
99 ECS traces by subtracting 546 nm from 520 nm absorbance changes. For such applications,  
100 the technique of “continuous ECS-indicated recording” *via* the dual-wavelength (550–520  
101 nm) accessory of the Dual-PAM-100 may be more appropriate (Klughammer et al., 2013).

102

103 *Saturating flash* - If a "single turnover saturating flash" is applied to the photosynthetic  
104 material, the ECS signal ( $\Delta A_{520\text{ nm}}$ ) is proportional to the amount of active (or open)  
105 photosystems,  $PS_0$ . The technical requirements are the following: the flash must be short  
106 (typically  $\leq 1\ \mu\text{s}$ , otherwise PSI can undergo a second turnover during the flash), and intense  
107 (typically  $\geq 1\ \text{mJ}$ , saturation is reached when twice as many photons have no significant effect  
108 ( $< 10\%$ ) to increase the signal). Pulsed lasers (flash-lamp pumped and frequency-doubled  
109 Nd:YAG lasers with dyes,  $\leq 10\ \text{ns}$  and  $\geq 30\ \text{mJ}$ ) typically meet these criteria. Here flashes at  
110 700 nm were delivered by an OPO pumped at 532 nm by a frequency-doubled Nd:YAG laser  
111 (Surelite II, Continuum). A saturating single-turnover flash gives the amount of "open" or  
112 "potentially active" photosystems: *i.e.* centers in the PA state if we call P the reduced form of  
113 the Primary donor and A the oxidized form of the Acceptor. The flash will close the centers,  
114 forming the  $P^+A^-$  state. In the dark, all centers are in the PA state, and during a continuous  
115 illumination some centers can be under the  $P^+A$  or  $PA^-$  states, which are other forms of closed  
116 centers.  $PS_0$  being the relative amplitude of the signal created by the flash is therefore the  
117 fraction of PA.

118

119 *Data treatment* - ECS kinetics were fitted by R-scripts. ECS rise and decay were respectively  
120 fitted to a linear model (initial flow rate  $I$ ) and first order exponential decay (pseudo steady-  
121 state flow rate  $J$ ), using the *nls* (Non-Linear Squares) function for the latter.

122

## 123 **Results**

124 Figure 1A shows the spectrum of light-induced absorbance changes ( $\Delta A$ ) 20  $\mu\text{s}$  after a  
125 saturating flash inducing an increase in trans-thylakoid  $\Delta\psi$ . Similarly as in (Witt, 1979), the  
126 spectrum shows a peak at 518 nm corresponding to carotenoid absorption shifting to longer  
127 wavelengths. After the flash,  $\Delta A$  decays to zero in about 100 ms, see Figure 1B (a). In the  
128 absence of ATPase, the ECS decay is much slower and the "slow electrogenic phase" of  
129 electron transfer in the low-potential chain (*b*-type hemes) of cytochrome *b<sub>6</sub>f* complex is  
130 observed, see Figure 2A. Subsequent flashes increase  $\Delta A_{520\text{ nm}}$  by the same amount, showing  
131 ECS is proportional to  $\Delta\psi$ . The amplitude of the flash-induced signal  $\Delta A_{20\ \mu\text{s}}$  is used as a  
132 simple calibration of 1 charge separated per photosystem (PS) and used for normalization of  
133  $\Delta A$ . Hereafter electron transfer rates (in  $\text{s}^{-1}$ ) are converted into electron flow rates (in  $\text{e}^- \text{PS}^{-1}$   
134  $\text{s}^{-1}$ ).

135

136 *Initial phase of ECS* - A continuous illumination induces multiphasic changes in  $\Delta A_{520\text{ nm}}$   
137 (Figure 1B). At the onset of illumination, the photosynthetic membrane is charged as a  
138 capacitor, the conversion of photons into electrons transferred across the membrane builds-up  
139  $\Delta\psi$ . Figure 1B (left) shows that ECS increases rather linearly in the first tens of milliseconds.  
140 In the absence of ATPase (Figure 2B),  $\Delta A_{520\text{ nm}}$  follows a straight slope over a much larger  
141 time range. Therefore, in order to estimate in the WT the initial photochemical flow rate  $I$  we  
142 need to fit the slope of the ECS rise on the first three datapoints ( $t \leq 10\text{ ms}$ ).  $I$  is proportional  
143 to the light intensity (Figure 2C). Inhibition of PSII has contrasting effects on the initial  
144 phase: when PSII is blocked only on the acceptor side, the initial flow rate is identical to the  
145 non-treated conditions (DCMU blocks the  $Q_A$  to  $Q_B$  electron transfer, but trans-membrane  
146 charge separation  $P_{680}^+Q_A^-$  still occurs); while when PSII is blocked at both donor and  
147 acceptor side (DCMU and hydroxylamine), the ECS rise is about twice slower (only PSI  
148 contributes to  $\Delta\psi$ ). This method can be used to assess the relative antenna size and the  
149 relative amounts of photosystems I and II. It also provides a way to calibrate the actinic effect  
150 of different light sources (of different wavelengths or bandwidths), converting incident  
151 photons  $\mu\text{mol}_{\text{photons}}\text{ m}^{-2}\text{ s}^{-1}$  into effective photons  $e^- \text{ PS}^{-1}\text{ s}^{-1}$ .

152

153 *Pseudo steady-state and decay phase* - After all photosystems were hit by photons (amplitude  
154  $\Delta A \sim 1$ , at  $t \sim 20\text{ ms}$  in Figure 1B, see inset on the left), the increase of ECS slows down, and  
155 a typical shoulder-peak-and-slow-descent is observed. The shape and amplitude of this curve  
156 is dependent upon the light intensity, the redox state (aerobic/anaerobic) or the nature of the  
157 sample (plant leaf or algal suspension), it is mostly influenced by the activation status of the  
158 chloroplast ATPase, the inter-conversion between  $\Delta\psi$  and  $\Delta\text{pH}$ , and possibly the activation of  
159 cyclic electron flow. At  $t \geq 3\text{ s}$  after the onset of illumination, ECS is in its "slow descent"  
160 phase, *i.e.* it does not vary much over time and it can be defined as a pseudo steady-state.  
161 When illumination stops, a sharp decay of ECS is observed (see Figure 1B right). It reflects  
162 the discharge of the membrane (efflux) that corresponded to the charge flow rate in the light,  
163 we denote  $J$  this flow at pseudo steady-state.

164

165 *A transmission coefficient for electrons* - In one quick measurement, ECS can therefore  
166 monitor the initial ( $I$ ) and pseudo steady-state ( $J$ ) rate of photosynthesis. To our knowledge,  
167 the only other way to get to these parameters is using fast-response bare  $\text{O}_2$  electrode with

168 modulated illumination (Haxo and Blinks, 1950; Joliot, 1965; Myers and French, 1960).  
169 Figure 3A shows that  $I$  increases linearly with irradiance. This is because when the  
170 photosystems are open, they are not limiting for electron transfer. In contrast  $J$ , here fitted to a  
171 Michaelis-Menten equation (solid line), reaches a plateau at  $V_{\max} = 88 \text{ e}^- \text{ s}^{-1} \text{ PS}^{-1}$ . It shows  
172 that photosynthesis saturates at high light intensities and suggests that photosystems are  
173 getting inactive during continuous illumination. The closure of photosystems can be assessed  
174 directly by adding a single turnover saturating flash in the slow descent at  $t = 3 \text{ s}$  (see  
175 Figure 1B). The flash gives a step increase with an amplitude equal to the fraction of open  
176 centers  $0 \leq \text{PS}_0 \leq 1$ .  $J$  is always smaller than  $I$ , and the ratio  $0 \leq J/I \leq 1$  is the yield of  
177 electron transfer. In other words  $J/I$  is the probability for an electron, initially transferred in a  
178 photosystem, to be steadily transported through the chain. This dimensionless parameter is a  
179 transmission coefficient.

180

181 *The slippery transmission of photosynthesis* - A photosynthetic apparatus with perfect  
182 transmission would give  $J/I$  equal to  $\text{PS}_0$ . In other words, for a first order rate reaction, the  
183 steady-state flow would be equal to the maximal rate (all centers open) in proportion to the  
184 fraction of centers remaining open at steady-state. In Figure 3B where  $J/I$  is plotted against  
185  $\text{PS}_0$ , data points do not align diagonally: the transmission coefficient is always smaller than  
186 the relative amount of open photosystems. It suggests that photosynthetic electron transfer is  
187 affected by the diffusion of the electron carriers. It is possible that a reduced PSII acceptor  
188 does not find its way towards an oxidized PSI donor before charge recombination occurs with  
189 a rate  $k_{\text{rec}}$ .

190 This problem of diffusion in the photosynthetic chain can be treated with a very economical  
191 model with only one parameter  $k_{\text{rec}}$ , a rate of charge recombination (Lavergne, 2009). We  
192 found that  $k_{\text{rec}}$  values comprised between 200 and  $700 \text{ s}^{-1}$  accounted for the gap between the  
193 measured transmission coefficient  $J/I$  and the fraction of centers remaining open in the light  
194  $\text{PS}_0$ . The solid and dotted lines correspond to an intermediary value  $k_{\text{rec}} = 350 \text{ s}^{-1}$ . A rate of  
195  $350 \text{ s}^{-1}$  ( $t_{1/2} = 2 \text{ ms}$ ) is in the range of charge recombination in PSII or PSI. At very low light  
196 intensities, when  $\text{PS}_0$  approaches 1 in Figure 3B, the vertical error bars become rather large,  
197 this is because of the division by  $I$  whose values tend to zero. Within experimental accuracy,  
198 the solid lines fit experimental data more satisfactorily than the dotted lines in Figure 3B, and  
199 also in Figure 3A where  $J$  rises more slowly than  $I$  (see Figure 3A inset). It shows that the  
200 maximum transmission coefficient,  $V_{\max} / K_m$  is reached when irradiance tends to zero, here  
201  $88 / 106 = 0.83$ . In other words, by analogy with mechanical engineering, the electron transfer

202 machinery has >17 % energy losses, even at idle speed. If one considers Photosystem I and  
203 Photosystem II as transmission shafts, the coupling in the photosynthetic chain compares to a  
204 friction belt, rubbing on the pulleys and dissipating mechanical energy as heat. This energy  
205 dissipation is accounted for in the  $k_{\text{rec}}$  parameter: frictions may encompass unpaired PSI-PSII  
206 antenna sizes (state transitions), non-photochemical quenching, non- $Q_B$ -reducing centers, as  
207 well as stochastic motion of electron carriers.

208  
209 *Increasing the frictions* – In the experiments shown in Figure 4, the wavelength of the actinic  
210 light was varied from 650 to 710 nm, in order to change the excitation balance between PSI  
211 and PSII. Yet, stronger light intensities were used at less absorbed wavelengths so that the  
212 actinic effect of the light was the same (same  $F_i$ , see Figure 4B). Figure 4A shows the ECS  
213 signal is much different under 680 nm excitation than at 710 nm. When both photosystems are  
214 excited,  $\Delta\psi$  builds up to a much higher level. This is not because of a slower decay of  $\Delta\psi$   
215 because in this instance  $\Delta\psi$  decays much faster,  $30 \text{ e}^- \text{ s}^{-1} \text{ PS}^{-1}$ , than when only PSI is excited,  
216  $8 \text{ e}^- \text{ s}^{-1} \text{ PS}^{-1}$  (Figure 4C). A “red drop” of the transmission coefficient  $J/I$  corresponds to the  
217 drop of the maximum quantum yield for  $\text{O}_2$  evolution (Emerson and Lewis, 1943). It is  
218 expected since these two parameters are homogenous: they both refer to a yield. Nevertheless,  
219  $\text{O}_2$  evolution drops more steeply than the transmission coefficient. It shows that far-red  
220 photons, absorbed by PSI and not by PSII, do not evolve  $\text{O}_2$  but sustain cyclic electron flow  
221 around PSI, although at a much slower rate than linear electron flow, as previously reported  
222 for DCMU treated samples (Alric, 2014, 2010; Maxwell and Biggins, 1976). Under such  
223 conditions  $\text{P}_{700}^+$  accumulates and PSI is closed while PSII centers stay potentially active and  
224 contribute to  $\text{PS}_0$ . When PSI and PSII excitation is unbalanced, the transmission coefficient in  
225 the chain decreases. In the long wave-range of photosynthesis, spinning Photosystem I  
226 transmission shaft while Photosystem II keeps still increases the frictions in the chain.

227

## 228 **Conclusion**

229 Half-saturation of photosynthesis was observed for a time delay of 0.02 s between short  
230 saturating flashes (Emerson and Arnold, 1932). In modern terms, it corresponds to an electron  
231 flow of  $1 / 0.02 = 50 \text{ e}^- \text{ s}^{-1} \text{ PS}^{-1}$ . In the present work, ECS measurements give a very similar  
232 value for half-saturation of photosynthesis:  $V_{\text{max}} / 2 = 44 \text{ e}^- \text{ s}^{-1} \text{ PS}^{-1}$  (see Figure 3A).  
233 Calibration of photosynthesis measurements on single turnover saturating flashes is therefore  
234 a way to compare results from lab to lab and from instrument to instrument. It also offers to  
235 precisely quantify the yield of photosynthesis. These "standard units" are independent from



236 the chlorophyll content (sample concentration, or thickness of the leaf) and are therefore also  
237 "portable" between photosynthetic organisms.

238

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242

### 243 **Author contribution**

244 C.M. performed experiments and analyzed data, J.A. designed research, analyzed data and  
245 wrote the manuscript.

246

### 247 **Data Availability Statement**

248 All data supporting the findings of this study are available within the paper.

249

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### 331 **Figure legends**

332

333 **Figure 1. ECS spectrum and kinetics in *Chlorella sorokiniana* intact cells, here measured**  
334 **with a Joliot-type spectrophotometer.**

335 **(A)** Spectrum of absorbance changes ( $\Delta A$ ), peaking at 518 nm, detected 20  $\mu\text{s}$  after a single  
336 turnover saturating flash.

337 **(B)** Typical kinetics of ECS, induced by a  $\sim 5$  s continuous red illumination  
338 ( $140 \mu\text{mol}_{\text{photons}} \text{m}^{-2} \text{s}^{-1}$ , 638nm, white bar). The signal was normalized on the amplitude of the  
339 single turnover flash (upward arrow) in the dark **(a)**. Insets show the fast phases of rise and  
340 decay in  $\Delta\psi$  at the onset **(b)** and offset **(d)** of light.  $I$  is the initial electron flow rate;  $J$  is the  
341 steady-state flow rate. The relative amount of photosystems remaining open during the light,  
342  $\text{PS}_{\text{o}}$ , is probed with a saturating flash **(c)**.

343

344 **Figure 2. Inactivating ATPase extends the range** where  $\Delta A_{520 \text{ nm}}$  increases with absorbed  
345 photons (SL8 mutant line).

346 **(A)** ECS following a train of 4 flashes (upward arrows). The ECS signal induced by the first  
347 flashes is of equal amplitude (see vertical bar:  $\Delta\psi$  amplitude generated by the transfer of one  
348 electron per photosystem). The decay of the signal is very slow (low membrane conductivity).

349 **(B)** ECS increases linearly with light duration. The linear range is larger in the ATPase-less  
350 strain (solid lines) than in WT (dotted lines). The initial rates, calculated between 0 and 10 ms  
351 are identical.

352 **(C)** The initial rate of ECS increases linearly with light intensity. 10  $\mu\text{M}$  DCMU does not  
353 block charge separation in PSII, but DCMU and hydroxylamine (10 mM) do, and decrease the  
354 initial rate.

355

### 356 **Figure 3. ECS light-response curves**

357 **(A)** Initial ( $I$ ) and steady-state ( $J$ ) electron fluxes plotted against light intensity.  $I$  is fitted to a  
358 straight-line,  $J$  to a Michaelis-Menten model, with  $V_{\text{max}} = 88 \text{ e}^{-} \text{PS}^{-1} \text{ s}^{-1}$  and  $K_{\text{M}} = 106 \text{ e}^{-} \text{PS}^{-1} \text{ s}^{-1}$   
359  $\text{s}^{-1}$  (solid line). The inset shows an expanded view at low-light conditions. The dashed line is  
360 when forcing  $K_{\text{M}} = V_{\text{max}}$  in the fit.

361 **(B)** Plot of the transmission coefficient  $J/I$  against the fraction of open photosystems  $\text{PS}_{\text{o}}$ .  
362 The solid line corresponds to a diffusive model with intrinsic losses, considering charge  
363 recombination at a rate  $k_{\text{rec}} = 350 \text{ s}^{-1}$ . The dashed line simulates the same diffusive model with  
364 maximum efficiency at low light ( $K_{\text{M}} = V_{\text{max}}$ , *i.e.* same initial slope for  $I$  and  $J$ , see (D) inset).  
365 The dotted diagonal corresponds to an "ideal" photosynthetic apparatus with perfect  
366 transmission (for  $k_{\text{rec}} = 0$ ).

367 In (A), (B) and Figure 1B, open symbols represent the same dataset (obtained at  $140$   
368  $\mu\text{mol}_{\text{photons}} \text{m}^{-2} \text{s}^{-1}$ ).

369

370 **Figure 4. ECS action spectra**

371 **(A)** ECS kinetics induced by 680 nm and 710 nm continuous light excitation.

372 **(B)** Light intensity (stronger at 710 nm than 680 nm) was adjusted to give the same initial  
373 rise.

374 **(C)** Steady-state electron flow is much slower under 710 nm light excitation specific to PSI.

375 **(D)** Photosynthesis action spectra: red drop in O<sub>2</sub> quantum yield (reproduced from (Emerson  
376 et al., 1957)) and transmission coefficient calculated from ECS ( $J/I$ ). Under 710 nm  
377 illumination P<sub>700</sub><sup>+</sup> is accumulated and PSI centers are closed.

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