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

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Highlight

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

Abstract

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

Keywords

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

Abbreviations

ECS, ElectroChromic Shift

pmf, proton-motive force

$\Delta\psi$, membrane potential

35 *I*, initial electron flow rate
36 *J*, steady-state electron flow rate
37 PS_o, open photosystems
38
39 **Introduction**
40 ECS signal was first reported as P515 (Duysens, 1954), an absorbance increase (ΔA) peaking
41 around 515 nm. This signal was attributed to a change in absorption of carotenoids (Witt,
42 1979), proportional to the membrane potential (Δψ). This signal was then used for the study
43 of proton flow through ATPase (Junge, 1970) and the "slow" electrogenic phase of cyt b_6f
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 Δψ and ΔpH (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

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

Materials and Methods

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

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

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

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

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

Results

Figure 1A shows the spectrum of light-induced absorbance changes (ΔA) 20 µs after a saturating flash inducing an increase in trans-thylakoid Δψ. Similarly as in (Witt, 1979), the spectrum shows a peak at 518 nm corresponding to carotenoid absorption shifting to longer wavelengths. After the flash, ΔA decays to zero in about 100 ms, see Figure 1B (a). In the absence of ATPase, the ECS decay is much slower and the "slow electrogenic phase" of electron transfer in the low-potential chain (*b*-type hemes) of cytochrome b_6f complex is observed, see Figure 2A. Subsequent flashes increase $\Delta A_{520\ nm}$ by the same amount, showing ECS is proportional to Δψ. The amplitude of the flash-induced signal $\Delta A_{20\ \mu s}$ is used as a simple calibration of 1 charge separated per photosystem (PS) and used for normalization of ΔA. Hereafter electron transfer rates (in s^{-1}) are converted into electron flow rates (in $e^- PS^{-1} s^{-1}$).

135

136 *Initial phase of ECS* - A continuous illumination induces multiphasic changes in $\Delta A_{520\ nm}$
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), $\Delta A_{520\ nm}$ 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 ($t \leq 10$ 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 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ into effective photons $e^{-}\ PS^{-1}\ s^{-1}$.
152

153 *Pseudo steady-state and decay phase* - After all photosystems were hit by photons (amplitude
154 $\Delta A \sim 1$, at $t \sim 20$ 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 ΔpH, and possibly the activation of
159 cyclic electron flow. At $t \geq 3$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 O_2 electrode with

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

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

This problem of diffusion in the photosynthetic chain can be treated with a very economical model with only one parameter k_{rec}, a rate of charge recombination (Lavergne, 2009). We found that k_{rec} values comprised between 200 and 700 s^{-1} accounted for the gap between the measured transmission coefficient J / I and the fraction of centers remaining open in the light PS_o. The solid and dotted lines correspond to an intermediary value $k_{rec} = 350$ s^{-1}. A rate of 350 s^{-1} ($t_{1/2}$ = 2 ms) is in the range of charge recombination in PSII or PSI. At very low light intensities, when PS_o approaches 1 in Figure 3B, the vertical error bars become rather large, this is because of the division by I whose values tend to zero. Within experimental accuracy, the solid lines fit experimental data more satisfactorily than the dotted lines in Figure 3B, and also in Figure 3A where J rises more slowly than I (see Figure 3A inset). It shows that the maximum transmission coefficient, V_{max} / K_m is reached when irradiance tends to zero, here 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_{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, Δψ builds up to a much higher level. This is not because of a slower decay of Δψ
215 because in this instance Δψ decays much faster, 30 $e^- s^{-1} PS^{-1}$, than when only PSI is excited,
216 8 $e^- s^{-1} PS^{-1}$ (Figure 4C). A "red drop" of the transmission coefficient J / I corresponds to the
217 drop of the maximum quantum yield for 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 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 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 P_{700}^+ accumulates and PSI is closed while PSII centers stay potentially active and
224 contribute to PS_o. 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 $e^- s^{-1} PS^{-1}$. In the present work, ECS measurements give a very similar
232 value for half-saturation of photosynthesis: $V_{max} / 2 = 44$ $e^- s^{-1} PS^{-1}$ (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

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

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Author contribution

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

Data Availability Statement

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

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Figure legends

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

(A) Spectrum of absorbance changes (ΔA), peaking at 518 nm, detected 20 µs after a single turnover saturating flash.

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

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

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

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

(C) The initial rate of ECS increases linearly with light intensity. 10 µM DCMU does not block charge separation in PSII, but DCMU and hydroxylamine (10 mM) do, and decrease the initial rate.

Figure 3. ECS light-response curves

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

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

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

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_2 quantum yield (reproduced from (Emerson
376 et al., 1957)) and transmission coefficient calculated from ECS (J / I). Under 710 nm
377 illumination P_{700}^{+} is accumulated and PSI centers are closed.

378