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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 $\mu g_{Chl}\ mL^{-1}$, 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 $e^-\ PS^{-1}\ s^{-1}$).

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

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

*A transmission coefficient for electrons* - In one quick measurement, ECS can therefore monitor the initial (*I*) and pseudo steady-state (*J*) rate of photosynthesis. To our knowledge, the only other way to get to these parameters is using fast-response bare $O_2$ 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 $V_{max} = 88\ e^{-}\ s^{-1}\ PS^{-1}$. 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 $t = 3$ s (see
175 Figure 1B). The flash gives a step increase with an amplitude equal to the fraction of open
176 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
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 $PS_o$. 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 $PS_o$, 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 $k_{rec}$.
190 This problem of diffusion in the photosynthetic chain can be treated with a very economical
191 model with only one parameter $k_{rec}$, a rate of charge recombination (Lavergne, 2009). We
192 found that $k_{rec}$ values comprised between 200 and 700 $s^{-1}$ accounted for the gap between the
193 measured transmission coefficient $J / I$ and the fraction of centers remaining open in the light
194 $PS_o$. The solid and dotted lines correspond to an intermediary value $k_{rec} = 350\ s^{-1}$. A rate of
195 350 $s^{-1}$ ($t_{1/2} = 2$ ms) is in the range of charge recombination in PSII or PSI. At very low light
196 intensities, when $PS_o$ 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

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

*Increasing the frictions* – In the experiments shown in Figure 4, the wavelength of the actinic light was varied from 650 to 710 nm, in order to change the excitation balance between PSI and PSII. Yet, stronger light intensities were used at less absorbed wavelengths so that the actinic effect of the light was the same (same $F_i$, see Figure 4B). Figure 4A shows the ECS signal is much different under 680 nm excitation than at 710 nm. When both photosystems are excited, $\Delta\psi$ builds up to a much higher level. This is not because of a slower decay of $\Delta\psi$ because in this instance $\Delta\psi$ decays much faster, 30 $e^- s^{-1} PS^{-1}$, than when only PSI is excited, 8 $e^- s^{-1} PS^{-1}$ (Figure 4C). A "red drop" of the transmission coefficient $J / I$ corresponds to the drop of the maximum quantum yield for $O_2$ evolution (Emerson and Lewis, 1943). It is expected since these two parameters are homogenous: they both refer to a yield. Nevertheless, $O_2$ evolution drops more steeply than the transmission coefficient. It shows that far-red photons, absorbed by PSI and not by PSII, do not evolve $O_2$ but sustain cyclic electron flow around PSI, although at a much slower rate than linear electron flow, as previously reported for DCMU treated samples (Alric, 2014, 2010; Maxwell and Biggins, 1976). Under such conditions $P_{700}^+$ accumulates and PSI is closed while PSII centers stay potentially active and contribute to $PS_o$. When PSI and PSII excitation is unbalanced, the transmission coefficient in the chain decreases. In the long wave-range of photosynthesis, spinning Photosystem I transmission shaft while Photosystem II keeps still increases the frictions in the chain.

## Conclusion

Half-saturation of photosynthesis was observed for a time delay of 0.02 s between short saturating flashes (Emerson and Arnold, 1932). In modern terms, it corresponds to an electron flow of $1 / 0.02 = 50$ $e^- s^{-1} PS^{-1}$. In the present work, ECS measurements give a very similar value for half-saturation of photosynthesis: $V_{max} / 2 = 44$ $e^- s^{-1} PS^{-1}$ (see Figure 3A). Calibration of photosynthesis measurements on single turnover saturating flashes is therefore a way to compare results from lab to lab and from instrument to instrument. It also offers to 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 $\mu mol_{photons}\ m^{-2}\ s^{-1}$).

**Figure 4. ECS action spectra**

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

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

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

**(D)** Photosynthesis action spectra: red drop in $O_2$ quantum yield (reproduced from (Emerson et al., 1957)) and transmission coefficient calculated from ECS ($J / I$). Under 710 nm illumination $P_{700}^{+}$ is accumulated and PSI centers are closed.