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Dear Editor

Please find here enclosed the mini-review “Electrochromism: a useful probe to study algal photosynthesis” by myself, Pierre Cardol, Cécile Breyton and Giovanni Finazzi.

This is an invited review for the special issue of Photosynthesis research entitled “Photosynthesis in eukaryotic algae”. The manuscript contains 5 figures.

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With best regards

Benjamin Bailleul
Electrochromism: a useful probe to study algal photosynthesis

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Abstract

In photosynthesis, electron transfer along the photosynthetic chain results in a vectorial transfer of protons from the stroma to the lumen space of the thylakoids. This promotes the generation of an electrochemical proton gradient ($\Delta \mu_{H^+}$), which comprises a gradient of electric potential ($\Delta \Psi$) and of proton concentration ($\Delta p$H). The $\Delta \mu_{H^+}$ has a central role in the photosynthetic process, providing the energy source for ATP synthesis, and being involved in many regulatory mechanisms. The $\Delta p$H modulates the rate of electron transfer and triggers de-excitation of excess light within the light harvesting complexes. The $\Delta \Psi$ is required for metabolites and protein transport across the membranes. Its presence also induces a shift in the absorption spectra of some photosynthetic pigments, resulting in the so called Electro Chromic Shift (ECS). In this review, we discuss the characteristics features of the ECS, and illustrate possible applications of it for the study of photosynthetic processes \textit{in vivo}. 
I. INTRODUCTION:

In plants, algae and photosynthetic bacteria, the primary electron donors and the electron acceptors of the photosynthetic complexes are located on opposite sides of the membrane. Light exposure results therefore in a charge separation across the thylakoids, due to photochemistry of the reaction centres (the photosystems, or PS) (Witt. 1979) and electron flow in the cytochrome \( b_{6f} \) complex. According to the Q cycle hypothesis, activity of this complex implies electron transfer in the “low potential chain” (Crofts et al. 1983), which spans the membrane owing to the location of the \( b_6 \) hemes in the complex (reviewed in Eberhard et al. 2008). Moreover, some electron flow steps involve the uptake or the release of protons in the aqueous phase. The consequent movement of electrons and protons through the membrane generates a proton motive force (pmf or \( \Delta \mu_{H^+} \)) which comprises an electric field (\( \Delta \Psi \)) and a proton concentration gradient (\( \Delta \rho \)). Ultimately, the energy of the \( \Delta \mu_{H^+} \) allows ATP synthesis by the activity of the ATP synthase complex.

I. 1 Electrochromism: the spectroscopic voltmeter. The presence of the electric field has also an effect on the photosynthetic pigments, the spectrum of which is modified owing to the Stark effect. This phenomenon, known as the electrochromism, has been largely studied in photosynthetic membranes since its first observation by Duysens in the 50’s (review in Witt. 1979). While exposure of chromophores to an electric field in solution results in various modifications in their physical properties, the main consequence in the case of pigments embedded in a lipid membrane is a shift of their absorption band. This effect, known as the “bandshift effect”, can be described as follows (Fig. 1A): absorption of a photon results in the energetic transition of the pigment from its ground state to an excited state. If these two states...
are characterised by different dipole moments (\(\bar{\mu}_e\) and \(\bar{\mu}_g\)) or polarisabilities (\(\alpha_e\) and \(\alpha_g\)), the energy difference (\(\Delta E\)) between the two states will be changed by the electric field (\(\vec{F}\)) (see e.g. Wraight et al. 1978; Lösche et al. 1988 for reviews), in a way described by the following relationship:

\[
\Delta \Delta E = - (\bar{\mu}_e - \bar{\mu}_g) \vec{F} - (\alpha_e - \alpha_g) \vec{F}^2
\]

The absorption spectrum of the molecule will be shifted (Fig. 1B), leading to the appearance of a “field-indicating absorption change”, the Electro Chromic Shift (ECS) signal.

\[
\Delta \lambda = \frac{\Delta \Delta E}{\hbar} = \frac{1}{\hbar} \left( (\bar{\mu}_e - \bar{\mu}_g) \vec{F} + (\alpha_e - \alpha_g) \vec{F}^2 \right)
\]

where \(\hbar\) is the Planck constant. If the shift is small when compared to the width of the absorption band (small perturbation), it is possible to generalize the equation above to the whole spectrum as:

\[
\Delta \varepsilon_{\lambda 0} = \left( \frac{\partial \varepsilon}{\partial \lambda} \right)_{\lambda 0} \Delta \lambda = - \frac{1}{\hbar} \left( \frac{\partial \varepsilon}{\partial \lambda} \right)_{\lambda 0} \left( (\bar{\mu}_e - \bar{\mu}_g) \vec{F} + (\alpha_e - \alpha_g) \vec{F}^2 \right)
\]

[1]

In this case, which is typical of photosynthetic systems, the theory of the electrochromic shift predicts two main features of the ECS: i.) The spectrum of the absorption change should follow the first derivative of the absorption band, and its shape should not respond to the field strength. Thus, the typical ECS spectrum obtained for a gaussian absorption band is a double-wave shape (Fig. 1B, C). In photosynthetic organisms, ECS spectra present several double-wave components (Fig. 2E, F), due to the superimposition of the ECS of different pigment absorption bands. ECS can provide therefore extremely precise information about the pigment
composition of a particular organism (see section II-1). ii.) The magnitude of the ECS is the sum of a linear and a quadratic function of the applied field (equation [1] and Fig 1C). In principle the ECS can be calibrated to provide absolute values of the electric field (discussed in Witt. 1979, see also Takizawa et al. 2007 for a more recent discussion). However, this calibration is extremely difficult, unless the two components (the linear and quadratic ones) are present at the same time (see section II-5).

In most of the photosynthetic systems, the ECS response shows however a linear response with respect to the intensity of the electric field. This feature of the ECS is extremely interesting for the purposes of this review because it means that the ECS is an intrinsic membrane voltmeter, which rapidly and linearly responds to changes in the membrane potential. Because the ECS can be measured spectroscopically in a non invasive way, this technique can be employed to monitor the characteristics of living plants and algae under physiological or modified conditions.

I. 2 ECS and photosynthesis. The use of the ECS signal to study the photosynthetic apparatus is often limited by the difficulty to correctly deconvolute this signal from other overlapping spectral changes. In vascular plants and green algae (Chlorophyceae, Trebouxyophyceae and Ulvophyceae), the maximal spectral change related to the ECS is around ~515 nm (Fig. 2A, B C and D). Therefore, any kinetic analysis of the field indicating absorption changes is carried out mostly at this wavelength. However, spectral changes at ~515 nm also reflect the formation and decay of triplet states of carotenoids (e.g. Kramer and Mathis. 1980). Moreover, accumulation of specific carotenoids in light exposed plants and green algae (due to
violaxanthin deepoxidation) results in a spectroscopic change at 505 nm (Sieferman and Yamamoto 1975) and in the apparition of an additional signal centred on 535 nm, which are linked to the building of the $\Delta pH$ (Heber. 1969). A correct deconvolution of the ECS signal thus requires a global analysis, including both spectral and kinetic measurements. Alternatively, the ECS signal can be differentiated from other signals by its sensitivity to ionophores, i.e. chemicals which increase the membrane permeability and therefore promote a fast relaxation of the electric field (Witt. 1979).

Despite the difficulty of correctly deconvoluting the ECS signal from field-independent spectral changes, previous studies have largely exploited this tool to assess the location and orientation of the pigments within the chlorophyll binding complexes, the origin of the electric field generated across the membranes, the topology of the primary electron donors and acceptors of PSII and PSI and the pathway for electron and proton transfer (reviewed in Witt. 1979).

It is possible to synchronize photosynthetic samples using repetitive flash spectroscopy, thus improving both the kinetic resolution and the signal to noise ratio of the ECS. The repetitive flash spectroscopy relies on the use of single turnover xenon or laser flashes. Kinetics analysis of the ECS signal in *Chlorella sorokiniana* cells (Joliot and Delosme. 1974) measured under repetitive flash regime has evidenced that the very fast rise of the ECS signal (<100 $\mu$s, “a” phase) reflects the rapid onset of the electric field due to charge separation by the PS (Fig. 3A). This is followed by a slower phase (“b” phase), occurring in the ms time range, which reflects cytochrome $b_{6}f$ complex activity. After completion of this phase, variable decay lifetimes are seen, which reveal the breakdown of the field by ionic flux. In native chloroplasts, this essentially stems from $H^{+}$ flux through the ATP synthase complex,
and variations in the decay lifetime (from a few milliseconds to several seconds) reveal different levels of activity of this enzyme.

The ECS signal measured under repetitive flash regime provides precise information about the photosynthetic complexes and about the pmf: i) the amplitude of the “a” phase is proportional to the number of active photosystems, and its change upon addition of specific PSII inhibitors allows obtaining the PSI/PSII stoechiometry (section II.2). ii) the “b” phase provides kinetic information on the cytochrome $b_6f$ and can be exploited to derive information on the size of the $\Delta \mu_{H^+}$ in the dark (Finazzi and Rappaport. 1998, section II.5) and iii) measurements of the rate of the decline phase of the ECS can be exploited to assess the activity of the ATP synthase in vivo (Lemaire and Wollman. 1989) or to evaluate the two components of the proton motive force in the dark (Joliot and Joliot. 2008, section II.4).

ECS measurements under continuous illumination can also be used to study alternative electron flow processes (reviewed in Eberhardt et al. 2008, section II.3) and to assess the relative extents of the light- induced $\Delta \Psi$ and $\Delta \rho pH$ in plants (Kramer et al. 2004; Cruz et al. 2005; Takizawa et al. 2007) and unicellular algae (Finazzi et al. 2006; Cardol et al. 2008, section II.4). In the following, we provide a more detailed description of the different applications of the ECS in vivo in the case of some unicellular algae.

II. APPLICATIONS

II. 1 Influence of the pigment composition and pigment orientation on the ECS.

Besides being a natural voltmeter, the ECS signal can be used as a non invasive tool to study the nature and environment of the photosynthetic pigments, provided that they undergo a band shift. This aspect is highlighted by the comparison between the
ECS features of plants leaves (Arabidopsis thaliana) and of the unicellular alga Ostreococcus tauri (Fig. 2D and 2E, respectively). In plants, the rather complex features observed in the blue-green part of the ECS spectrum reflect the contribution of chlorophylls a and b and of carotenoids to the overall shift (e.g. Schmidt et al. 1971). On the other hand, the peaks observed in the red region of the spectrum (Witt. 1979) are more symmetric, consistent with the involvement of the sole chlorophyll molecules in this region. In O. tauri, the blue green ECS spectrum is a typical carotenoid bandshift, suggesting that chlorophylls are probably less sensitive to the applied field in this alga. Therefore, the ECS features in Ostreococcus can be used to study the nature and orientation of carotenoids in the photosynthetic membranes.

The ECS spectrum of Ostreococcus shows two major peaks at ~505 and ~530 nm, located asymmetrically to a center at 510- 515 nm (Fig. 4D), which clearly corresponds to the maximum difference in the absorption spectra of the light harvesting complexes of Arabidopsis (LHClI) and Ostreococcus (LHCP) (Fig. 4B, C). This suggests that an Ostreococcus-specific carotenoid (e.g. prasinoxanthin the most abundant carotenoid in this alga, Six et al. 2005), is responsible for the ECS shift. The spectrum measured in Ostreococcus is also illustrative of the principle that the ECS features reflect the environment experienced by the field-responding pigments. While, in plants, the light- induced generation of an electric field results in the red shift of the spectrum, a transition towards the blue is observed in Ostreococcus. This suggests that the orientation between the field and the transition moment of the carotenoid is antiparallel (see equation [1]), i.e. a rather unusual configuration for photosynthetic organisms (e.g. Lösche et al. 1988). This finding implies that the spatial arrangement of carotenoids within the LHCP complex must be very different from what is known in LHClI (Liu et al. 2004), consistent with previous conclusions
based on linear and circular dichroism analysis of the LHCP complex from the prasinophyte *Mantoniella squamata* (Goss et al. 2000).

**II 2. Application of the ECS to study acclimation of the photosynthetic apparatus.**

The non invasive character of the ECS, together with the possibility to synchronize the photosynthetic turnover, allow deriving information about changes in the architecture of the photosynthetic chain, following acclimation to environmental stimuli. Changes in the photosynthetic apparatus are typically observed in photosynthetic organisms subjected to nutrient deficiencies, where photosynthesis decreases due to changes in the activity and stoichiometry of the photosynthetic complexes (Merchant et al. 2006). Photosynthetic acclimation to nutrient availability is particularly well characterised upon iron starvation, which changes both the light absorption and electron flow capacities of the cells. Iron is of primary importance in biological systems because it is a central constituent of hemes and iron-sulfur clusters. The activity of many types of enzymes, particularly the ones involved in energy conversion processes, relies on these redox centers. Therefore, several mechanisms exist to insure iron homeostasis in plants and algae (for reviews see Curie et al. 2003; Briat et al. 2007). Iron mobilization is critical for micro algae in the iron-limited open ocean and for terrestrial plants, which are exposed to limited iron bio-availability because the prominent form of iron in soil in the presence of oxygen, Fe$^{3+}$, is poorly soluble at neutral and alkaline pH. In photosynthesis, PSI has the highest iron content (12 Fe per reaction centre) and is therefore particularly sensitive to iron deficiency. Deprivation of this metal induces changes at the level of both PSI light harvesting apparatus and reaction centre. In cyanobacteria, Fe starvation
induces the expression of IsiA, a chlorophyll binding protein similar to CP43, which binds to the PSI cores (Singh and Sherman. 2007). Similar changes are observed in the halotolerant eukaryotic alga *Dunaliella salina* (Varsano et al. 2006), where the light harvesting protein homolog Tidi accumulates in iron limited conditions. While modifications in PSI antenna are also observed in Fe-deficient *Chlamydomonas reinhardtii* cells (Moseley et al. 2006), no significant changes in the PSI antenna of plants have been reported so far (Timperio et al. 2007). In general, the most important effect of iron deprivation is a marked drop in PSI centers, relative to other molecular constituents of the photosynthesis apparatus, which results in a reduced capacity to reoxidize the plastoquinone pool in the light. The drop in PSI complexes is particularly severe in cyanobacteria, leading in some instances to a four time decrease in the PSI/PSII ratio (e.g. Guikema and Sherman. 1984; Sandstrom et al. 2002). Large changes in PSI content have also been observed in photosynthetic eukaryotes, including *Chlamydomonas* (Moseley et al. 2006) and centric diatoms (Strzepek and Harrison. 2004). Recently, the employment of the ECS approach has allowed a precise quantification of the PSII/PSI stoichiometry in Fe starved unicellular algae including diatoms (Allen et al. 2008), Ostreococcus (Cardol et al. 2008) and *Chlamydomonas* (Petritsos et al. 2009). Detecting changes in the PSII/PSI stoichiometry with the ECS measurement requires single turnover flash spectroscopy. In conditions where all the active photosystems perform one charge separation, i.e. after excitation by a saturating laser flash, the “a” phase of the ECS is indicative of the total number of active reaction centres in the chloroplasts. Conversely, the addition of DCMU and hydroxylamine to completely block the PSII turnover makes the flash induced ECS signal proportional to the sole number of PSI. Thus, PSII contribution can be calculated from the decrease in the signal amplitude
upon the addition of DCMU and hydroxylamine, whereas PSI can be estimated as the fraction of the signal that is insensitive to these inhibitors (see Fig. 3B). At variance with other techniques employed before, as biochemical assessments of complex subunits (e.g. Anderson et al. 1995), evaluations of oxygen evolution vs P700 oxidation extents (Melis, 1989) and EPR measurements (Danielsson et al. 2004), the ECS approach provides a more reliable quantification procedure (e.g. Chow et al. 2000; Fan et al. 2007) as it does not require any normalisation between the different parameters employed to assess the PSII and PSI amounts. On the other hand, a correct estimate of the PSI/PSII ratio requires the utilisation of laser flashes, to avoid occurrence of double PSI turnovers, which otherwise leads to over-estimation of this complex. In most photosynthetic organisms, recovery of photo-active PSI centers is limited by the rate of re-reduction of P700⁺, the primary electron donor of PSI. This process can be fast, however, if plastocyanin and/or cytochrome c₆ (the soluble PSI donors) are pre bound to the reaction centre. Pre bound PSI donors can reduce P700⁺ in less than ten microseconds (e.g. Delosme. 1991), i.e. a time that is shorter than the average lifetime of commercial xenon sources (30-50 microseconds). A xenon flash can therefore excite a PSI with a soluble donor bound, inducing a first charge separation, and then, after P700⁺ rereduction by bound PC or cyt c₆, excite it a second time during the long flash (especially if the flash intensity is high). In green algae, it has been estimated that the maximum extent of double PSI turnover in saturating light is ca 30-40% (Farah et al. 1995).

II. 3. Application of the ECS approach to study alternative electron flow processes. Measurements of the ECS signal under continuous illumination are most suitable to study the overall function of the photosynthetic chain. To measure the ECS in continuous light, it is necessary to deconvolute this signal from other
overlapping optical changes (Witt 1979). As discussed in the introduction, this can be achieved thanks to the different relaxation times of the different signals: while triplet relaxation is almost instantaneous, both the 505 and the 534 absorption changes are much slower than the ECS (see above). Thus techniques have been developed to study the ECS in continuous light, including the so called DIRK (dark interval relaxation kinetics, Sacksteder and Kramer. 2000) or the “dark pulse” one (Joliot and Joliot. 2002). In both cases, the ECS signal is probed at appropriate wavelengths, both when steady-state photosynthesis is established by continuous illumination and when light is switched off for a short time (in general less than 1 minute). In steady state, the contribution of PSI, PSII, cytochrome \( b_{6f} \) and of ion leak through the membrane is not distinguishable on a kinetic base. The overall rate of membrane potential formation \( V_{ph} = R_{ph} + R_{b6f} - R_{leak} \) is the sum of the photochemical rate of membrane potential formation \( R_{ph} \), of the cyt \( b_{6f} \) specific rate \( R_{b6f} \), and of the rate \( R_{leak} \) of ion leaks through the membrane (Fig. 5A). When the light is switched off, \( R_{ph} \) immediately falls to zero whereas \( R_{leak} \) and \( R_{b6f} \) are unchanged. Therefore, \( R_{ph} \) can be evaluated form the difference \( (S_L - S_D) \) between the slopes of the ECS signal measured immediately before \( (S_L) \) and after \( (S_D) \) the light is switched off (Joliot and Joliot. 2002). The possibility to estimate the photochemical rates of PSI and PSII opens the possibility to study electron diversion to other sinks than CO\(_2\). In steady state, electron transfer mainly stems from linear electron flow (LEF), which involves both PSII and PSI activities, and results in CO\(_2\) assimilation. However, the Calvin cycle is mostly inactive at the onset of illumination (Ort and Baker. 2002; Johnson. 2005), and linear electron flow is therefore limited by the low rate of NADPH reoxidation. A significant fraction of the photo- generated electrons is available therefore for other processes during this phase of photosynthesis. Among them,
cyclic electron flow (CEF) activity is considered to be predominant in plants (e.g. Joliot and Joliot. 2006). The high efficiency of the CEF is interpreted as the consequence of a redox modulation of the relative efficiencies of the linear and cyclic processes (see Allen. 2003).

Using the DIRK approach, the fraction of reaction centers involved in CEF can be taken into account while estimating the membrane potential formation rate due to photochemistry (Joliot and Joliot. 2002):

\[
R_{\text{ph}} = R_{\text{PSII}} + R_{\text{PSI linear}} + R_{\text{PSI cyclic}}
\]

with
\[
R_{\text{PSI linear}} = R_{\text{linear}} = R_{\text{PSII}}
\]

therefore,
\[
R_{\text{PSI cyclic}} = R_{\text{ph}} - 2 \times R_{\text{PSII}}.
\]

As stated above, \( R_{\text{ph}} \) is derived from ECS measurements. \( R_{\text{PSII}} \) can be estimated from fluorescence measurements using the Genty parameter (Genty et al. 1990): \( R_{\text{PSII}} = k_{\text{PSII}} \times (\text{Fluo}_{\text{ss}} - \text{Fluo})/(\text{Fluo}_{\text{max}} - \text{Fluo}_0) \), where \( \text{Fluo}_{\text{max}} \) is the maximum fluorescence emission by PSII, \( \text{Fluo}_{\text{ss}} \) is the steady state emission, \( \text{Fluo}_0 \) is the minimum fluorescence emission in dark adapted samples, and \( k_{\text{PSII}} \) is the photochemical rate of photosystem II.

Previous work has established that the Calvin cycle requires ATP and NADPH in a stoichiometry of 1.5, i.e. a ratio that cannot be entirely fulfilled by the sole operation of LEF, which provides an insufficient proton to electron balance when compared with the stoichiometry of \( H^+ \) required to fuel ATP synthesis by the chloroplast ATP synthase (Allen. 2003). Thus, alternative electron flow could contribute to additional proton gradient formation, and from there to additional ATP synthesis to re-equilibrate the ATP/NADPH stoichiometry for proper carbon
assimilation (review in Eberhardt et al. 2008). Besides cyclic electron flow, the water to water cycle (Mehler. 1951) could also contribute to the synthesis of “extra ATP” in cyanobacteria (e.g. Zhang et al. 2009) and unicellular algae (e.g. Johnson et al. 2010). In particular, a different version of the water to water cycle has been recently observed in marine *Synechococcus* under Fe starvation (Bailey et al. 2008) and in a particular *Ostreococcus* ecotype (*Ostreococcus RCC809*, Cardol et al. 2008), where a significant fraction of electrons generated by PSII are consumed by a plastid plastoquinol terminal oxidase (probably PTOX).

This alternative electron flow, which bypasses PSI activity, should also lead to the generation of a proton gradient without NADPH, by boosting PSII activity. Indeed, the combined oxidation of water by PSII and of plastoquinol by PTOX should lead to the vectorial transfer of H⁺ from the stroma to the luminal space (e.g. Eberhardt et al. 2008) This possibility was experimentally tested using the ECS tool in the case of *Ostreococcus RCC809*. In this strain, inhibition of PSII activity with DCMU completely abolished the generation of the ECS, indicating that the CEF is negligible. Conversely, the inhibition of the cytochrome *b*₆*f* with the specific inhibitor dibromothymoquinone (DBMIB), although blocking both linear and cyclic electron flows, allowed a substantial fraction of this signal to be maintained. Further addition of propylgallate, a PTOX inhibitor, suppressed the DBMIB insensitive field generation, indicating that PTOX contributes to the generation of a pmf in this alga.

By introducing a new term related to PTOX activity in equation [2], one obtains

\[ R_{ph} = R_{PSII-linear} + R_{PSII/PTOX} + R_{PSI-linear} + R_{PSI-cyclic} \]

Considering that \( R_{PSI-cyclic} \approx 0 \), and that \( R_{PSI-linear} = R_{PSII-linear} \) one obtains

\[ R_{PSII/PTOX} = R_{ph} - 2 \cdot R_{PSII-linear}. \]
Knowing that $R_{PSII/PTOX} \sim 0.5 R_{ph}$ (Cardol et al. 2008), one obtains $R_{PSII/PTOX} \sim 2$ $R_{PSII-linear}$, i.e only 1/3 of the electrons from PSII are directed to PSI, in agreement with the estimates of the PSII/PSI ratio in *RCC809* (~3/1, Cardol et al. 2008). This suggests that linear flow is strictly limited by the PSI availability in this alga. Therefore, it is tempting to propose that diversion of electrons to oxygen downstream of PSII, but before PSI, may reflect a common and compulsory strategy in marine phytoplankton to bypass the constraints imposed by light and/or nutrient limitation and allow successful colonization of the open-ocean marine environment.

**II 4. The ECS approach to study the pmf composition under illumination.** When the DIRK method is extended to longer dark period, an interesting phenomenon is observed: after the first decay, a slower rise phase is seen which leads to an inversion of the ECS signal (e.g. Cruz et al. 2001, see Fig. 5B in the case of the green alga *Chlorococcus ellipsoideum*). This inversion has been interpreted in terms of the different rate of relaxation of the two components of the pmf. After illumination, the electric component of the proton motive force should relax faster than the $\Delta pH$, due to the low dielectric constant of the thylakoid membranes (Vredenberg. 1976), the high $H^+$ buffering capacity of the lumen (Junge and McLaughlin. 1987), and the slow rate of charge redistribution along the membranes (see Cruz et al. 2001 for a further discussion). In the fast phase, the pmf relaxation to its dark adapted state is allowed by the net transfer of positive charges from the lumen to the stroma. This would decrease the $\Delta \Psi$ component (and ECS signal) to a level below its dark adapted level, to compensate for the sustained light generated $\Delta pH$. Conversely, the slow rise phase would reflect the kinetics of the slow relaxation of the buffered $\Delta pH$, continuously compensated by a flux of counter-ions (i.e. an increase of the ECS
signal), until both $\Delta pH$ and $\Delta \Psi$ have reached their dark-adapted levels (Kramer et al. 2004).

Based on the overall amplitude of the total and of the inverted ECS signals, attempts have been made to estimate the partitioning between the light-induced $\Delta pH$ and $\Delta \Psi$ components of the pmf in plants and green algae (Fig. 5B, Kramer et al. 2004, Cruz et al. 2005). These estimates have indicated that the size of the $\Delta pH$ is by far lower than what was previously estimated based on in vitro measurements (e.g. Rumberg. 1969). Thus, Kramer and colleagues have proposed a ‘moderate’ lumen pH hypothesis (Sacksteder and Kramer. 2000), which assumes that the lumen pH is maintained in a range where it can regulate light capture while not damaging the photosynthetic apparatus. The predicted lumen pH should range between 7.8 and 5.7 (Kramer et al. 2004). On the other hand, measurements of the ECS in the dark (see section II.5) have revealed the existence of a pmf across the thylakoid membranes in both dark adapted algae (Finazzi and Rappaport. 1998) and plants (Joliot and Joliot. 2008) which comprises a $\Delta pH$ of 0.5-1 unit (Heldt et al. 1978). These findings may provide a mean to reconcile the in vitro and in vivo $\Delta pH$ estimates. Indeed, the sum of the dark- plus the light-induced proton gradient in vivo closely resembles the pH gradient estimated in vitro.

**II 5. Quadratic components of ECS allow for the measurement of a pre-existing proton gradient.** Because of the linearity between the ECS and the applied field, the ECS techniques only allow measuring changes of the pmf but not its absolute value, as required to study ATP synthesis and regulation of the photosynthetic process.

But, as discussed above, the ECS signal can in principle be calibrated, leading to the estimate of the absolute value of the $\Delta \Psi$ in the light (Witt 1979, Joliot and Joliot
Indeed, the amplitude of the spectral shift undergone by a pigment when submitted to an electric field \( F \) is proportional to \( F \) or \( F^2 \) depending on the polarization state of the pigment (equation [1]). The amplitude of the linear ECS response \( (\Delta ECS_{\text{lin}}) \) observed upon a flash-induced increase of the electric field \( (\Delta F, \text{due to charge separation}) \) is constant, independent of the value of the field preexisting the perturbation (Fig. 1C). At the contrary, the amplitude \( (\Delta ECS_{\text{quad}}) \) of the quadratic ECS response depends on the value of the preexisting field \( (F_0) \). It follows that, in organisms where the quadratic response is not negligible with respect to the linear one, the amplitude of the flash-induced quadratic ECS can be used to probe the absolute value of the electric field. This has been experimentally evidenced in \textit{Chlorella} and \textit{Chlamydomonas reinhardtii} mutants devoid of the light harvesting complexes. In these mutants, most of the pigment binding proteins are absent and both the linear and quadratic probes are observed, at variance with wild type cells, where the linear response is largely predominant (Joliot and Joliot 1989).

Using these mutants, the amplitude of the electric component (the \( \Delta \Psi \)) of the large electrochemical gradient built in darkness has been evaluated and found to be twice as small as the \( \Delta \text{pH} \), assessed from the kinetics of the “b” phase (Finazzi and Rappaport. 1998). The amplitude of the pmf in the dark (~110-140 mV) fits well with estimations based on the ATP/ADP ratio measured in these algae, suggesting that it is built at the expenses of ATP hydrolysis by the ATP synthase-ATPase complexes. A pmf of reduced size was found in anaerobic (respiration inhibited) conditions compared to aerobic (respiration active) conditions, confirming that the ATP is of respiratory origin. The dark pmf could have several functions \textit{in vivo}: it could help maintaining a constitutive amount of activated ATP synthase complexes, thereby facilitating ATP synthesis upon illumination. Indeed, activation of the ATP synthase
upon a light transition is mediated by the building of a pmf (Junesh and Graber. 1985). The dark $\Delta \mu_{H^+}$ could also allow maintaining the enzymes involved in the xanthophylls cycle (the activation of which is $\Delta \rho H$ dependent) in a partially active state. This would facilitate photo-protection during a dark to light transition, by speeding up the onset of non photochemical quenching of absorbed photons (NPQ, Horton et al. 1996; Niyogi. 1999). Finally, the existence of a $\Delta \mu_{H^+}$ in the dark could provide the energy source for protein import in the dark, thus maintaining an active photosynthetic apparatus (reviewed in Colinson et al. 2003).

III. PERSPECTIVES

As discussed above, employment of the ECS tool allows studying the energetics of the chloroplast in vivo, opening the possibility to investigate several processes related to plant physiology (the balance between ATP synthesis and generation of reducing equivalents; chloroplast assembly and degradation, acclimation to environmental conditions, etc.). Obviously, this approach can be applied only to organisms which display an ECS signal. As a general rule, photosynthetic organisms possessing intrinsic light harvesting complexes should show an ECS band shift when exposed to illumination. This is true in the case of green algae and diatoms (Fig. 2). Recent measurements of the ECS in the pennate diatom Phaeodactylum tricornutum indicated the coexistence of linear and quadratic probes within the same light harvesting complex (Bailleul and Finazzi, unpublished). This finding is of great advantage, as the presence of pigments responding to $F$ and $F^2$ allows assessing the absolute value of the $\Delta \Psi$ and $\Delta \rho H$ and therefore to follow the dynamics of the pmf in a living organism.
Figure legends

Figure 1. **Schematic representation of the mechanism of electrochromism. A.** Application of an electric field induces a change of the energy difference between the ground and the excited state of a chromophore. **B.** This results in a shift of the absorption band, which leads to the appearance of a double wave shape, typical of the Electrochromic Shift (open circles). **C.** Dependency of linear (red) and quadratic (blue) components of the ECS upon the field, and effect of the pre-existing field on the flash-induced ECS signals.

Figure 2. **ECS signals in different photosynthetic organisms: A. Chlorella mirabilis, B. Cephaleuros parasiticus. C. Scenedesmus obliquus D. Ostreococcus tauri E. Arabidopsis thaliana F. Phaeodactylum tricornutum.** Algae and or leaves were illuminated with continuous light, and the ECS spectra a presented as the light minus the dark signal.

Figure 3. **Kinetics of the ECS measured in intact cells of the green alga Chlamydomonas reinhardtii** upon excitation with a saturating laser pulse. Cells were adapted to anaerobic conditions (with nitrogen bubbling) to slow down membrane potential decay through the ATP, and better evidence the slow ECS rise in the ms range (e.g. Joliot and Delosme, 1974). **A.** The fast rise phase (red) corresponds to PSI and PSII charge separation, which occurs on an unresolved timescale. The ms time range rise phase corresponds to electron flow in the cytochrome b chain of the cytochrome $b_{6f}$ complex (blue). The ECS decay (green) is due to charge leakage through the membrane, mainly $H^+$ via the ATP synthase. **B.** Application of flash- induced ECS signals to calculate PS stoechiometries.
Inhibition of PSII activity (right panel, addition of DCMU) results in a decrease of the amplitude of this phase. The relative extent of the amplitude decrease is directly proportional to the PSI/ (PSI + PSII) ratio, and allows to estimate the stoichiometry of functional reaction centres.

Figure 4 Absorption spectrum of the LHC and PSI bands from vascular plants (Arabidopsis thaliana leaves) and Ostreoccus, ecotype OTH95. A. Non-denaturating, 10% SDS-PAGE electrophoresis gel run at 4°C of OTH95 cells (Ot), and Arabidopsis thaliana (At) thylakoids. Thylakoids were prepared using standard procedures and 10 µg Chl of 2% SDS solubilised material were loaded. The difference in apparent molecular weight between LHCP and LHCII reflects a stronger resistance of LHCP trimer to SDS denaturation, as LHCP and LHCII migration on either BN- or denaturing SDS-PAGE is comparable (not shown). B Absorption spectrum of the antenna bands in the 400-700 nm region. C. Absorption spectrum difference between the A. thaliana and OTH95 light harvesting complexes. Absorption spectra were normalised at 670 nm before calculating their difference spectrum. f.p. free pigments. C. ECS spectrum of OTH95, measured in living cells upon excitation with a saturating laser pulse. The light minus dark spectrum is shown.

Figure 5. The ECS signal allows measuring the turnover of the photosynthetic chain in continuous light. A. By measuring the transient changes of the ECS under steady-state illumination or upon switching the light off, it is possible to accurately estimate electron transfer kinetic and thermodynamic parameters, as shown. See text for further details. B. ECS spectrum (left panel) and ECS changes kinetics (right
panel) in *Chlorococcum ellipsoides*. After switching the light off, a fast decay followed by a slower rise phase are seen which have been interpreted in terms of the different rate of relaxation of the two components of the pmf, and lead to an inversion of the ECS signal. Black and white bars represent dark and light phases, respectively.
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A. Energy levels with and without an electric field. The ground state is shown as \((\mu_g, \alpha_g)\) and the first excited state as \((\mu_e, \alpha_e)\). The energy change \(\Delta E\) with an electric field of \(\Delta \lambda\) is shown.

B. Extinction coefficient \(\epsilon\) vs. wavelength \(\lambda\) (nm) with and without an electric field. The solid line represents no electric field, and the dashed line represents with electric field.

C. The ratio of change in extinction coefficient \(\Delta \epsilon / \epsilon\) with quadratic and linear models. The difference in extinction coefficient \(\Delta ECS\) is shown for both linear and quadratic models with changes in field strength \(\Delta F\).
Bailleul et al.,
Figure 2

Click here to download Colour figure: ECS-Figure2.ppt
Bailleul et al., Figure 5

A

\[ S_I = R_{ph} + R_{b6f} - R_{leak} \]

\[ R_{ph} + R_{b6f} \]

\[ R_{leak} \]

\[ S_d = R_{b6f} - R_{leak} \]

B

\[ \Delta \varepsilon / \varepsilon \times 10^3 \]

\[ \lambda \text{ (nm)} \]

\[ \Delta \Psi \]

\[ \Delta pH \]

Click here to download Colour figure: ECS-Figure5.ppt