

DARWIN REVIEW

Linking chlorophyll *a* fluorescence to photosynthesis for remote sensing applications: mechanisms and challenges

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Received 14 February 2014; Revised 21 March 2014; Accepted 31 March 2014

Abstract

Chlorophyll *a* fluorescence (ChlF) has been used for decades to study the organization, functioning, and physiology of photosynthesis at the leaf and subcellular levels. ChlF is now measurable from remote sensing platforms. This provides a new optical means to track photosynthesis and gross primary productivity of terrestrial ecosystems. Importantly, the spatiotemporal and methodological context of the new applications is dramatically different compared with most of the available ChlF literature, which raises a number of important considerations. Although we have a good mechanistic understanding of the processes that control the ChlF signal over the short term, the seasonal link between ChlF and photosynthesis remains obscure. Additionally, while the current understanding of *in vivo* ChlF is based on pulse amplitude-modulated (PAM) measurements, remote sensing applications are based on

Abbreviations: A_G , gross photosynthetic rate (e.g. $\mu\text{mol CO}_2$ fixed by the Calvin–Benson cycle $\text{m}^{-2} \text{s}^{-1}$); A_N , net photosynthetic rate (e.g. $\mu\text{mol CO}_2$ exchanged at the leaf scale $\text{m}^{-2} \text{s}^{-1}$); APAR, the flux of photosynthetically active radiation absorbed by plants (e.g. $\mu\text{mol photons m}^{-2} \text{s}^{-1}$); a_i , a_{ii} , relative absorption cross-section areas of PSI and PSII populations; range from zero to one; CET, cyclic electron transport; ChlF, chlorophyll *a* fluorescence; ETR, electron transport rate through PSII ($\mu\text{mol electrons m}^{-2} \text{s}^{-1}$), equivalent to LET; F , fluorescence signal emanating from a leaf, as in Equation 11; f APAR, fraction of incoming PAR absorbed by vegetation; range from zero to one; F_{ii} , F_{iIM} , prevailing and maximal fluorescence signal at the level of PSII, respectively; $F_{PSI}(\lambda_{em})$, function that accounts for the shape of the fluorescence emission spectra in PSI; $F_{PSII}(\lambda_{em})$, function that accounts for the shape of the fluorescence emission spectra in PSII; F_R , F_{FR} , fluorescence measured in the red or far-red region, respectively; F' , F'_M , prevailing and maximal fluorescence signal as measured with PAM fluorometry (relative units, e.g. sensor mV output); F_0 , F_M , minimal and maximal fluorescence signal as measured with PAM fluorometry in the dark and after a period of dark acclimation (relative units, e.g. sensor mV output); ΦF , quantum yield of fluorescence (quanta emitted/quanta absorbed); ΦF_i , ΦF_{ii} , quantum yield of fluorescence in PSI and PSII, respectively; ΦP , quantum yield of photochemistry in PSII (electrons transported/quanta absorbed); ΦP_{max} , maximum quantum yield of photochemistry in PSII obtained after dark acclimation or during the night; GPP, gross primary productivity (e.g. total $\mu\text{mol CO}_2$ assimilated by plants $\text{m}^{-2} \text{s}^{-1}$); k_{CI} , rate constant of excitation energy transfer between neighbouring PSII units that denotes the degree of excitonic connectivity (s^{-1}); k_D , rate constant of basal or constitutive thermal energy dissipation (s^{-1}); k_F , rate constant of chlorophyll fluorescence emission (s^{-1}); k_{ISQ} , rate constant of intersystem crossing (s^{-1}); k_{NPQ} , rate constant of regulated thermal energy dissipation or NPQ (s^{-1}); k_P , overall rate constant of photochemistry (s^{-1}); k_{PSII} , intrinsic rate constant of photochemistry in open and fully functional reaction centres (s^{-1}); k_{RCI} , rate constant of thermal energy dissipation by closed PSI (s^{-1}); k_T , rate constant of energy transfer between neighbouring pigments (s^{-1}); LET, linear electron transport; LUE, light use efficiency of photosynthesis ($\mu\text{mol CO}_2$ assimilated/quanta absorbed); NPQ, non-photochemical quenching of excitation energy via regulated thermal energy dissipation; *NPQ* (in italics), a fluorescence parameter to quantify NPQ capacity, proportional to $k_{NPQ}/(k_D+k_F)$; PQ, photochemical quenching of excitation energy via electron transport; *PQ* (in italics), a fluorescence parameter to quantify PQ capacity, proportional to $k_P/(k_D+k_F)$; PR, photorespiration rate ($\mu\text{mol CO}_2$ photorespired $\text{m}^{-2} \text{s}^{-1}$); PRI, photochemical reflectance index; PSI, photosystem I; PSII, photosystem II; q , fraction of open and functional reaction centres; range from zero to one. q_L , the photochemical quenching parameter based on a lake model assumption, an estimate of q ; q_P , the photochemical quenching parameter based on a puddle model assumption, an estimate of q ; R_d , the rate of leaf-level mitochondrial day respiration (e.g. $\mu\text{mol CO}_2$ respired $\text{m}^{-2} \text{s}^{-1}$); SIF, solar-induced fluorescence (e.g. in $\text{W m}^{-2} \text{s}^{-1} \text{nm}^{-1}$), also sun-induced fluorescence.

the measurement of the passive solar-induced chlorophyll fluorescence (SIF), which entails important differences and new challenges that remain to be solved. In this review we introduce and revisit the physical, physiological, and methodological factors that control the leaf-level ChlF signal in the context of the new remote sensing applications. Specifically, we present the basis of photosynthetic acclimation and its optical signals, we introduce the physical and physiological basis of ChlF from the molecular to the leaf level and beyond, and we introduce and compare PAM and SIF methodology. Finally, we evaluate and identify the challenges that still remain to be answered in order to consolidate our mechanistic understanding of the remotely sensed SIF signal.

Key words: Gross primary production, GPP, leaf level, photosystem II, photosystem I, PSII, PSI, photosynthesis dynamics, pulse amplitude modulation, PAM, PSII connectivity, remote sensing, solar-induced fluorescence, sun-induced fluorescence, SIF.

1. Introduction

Photosynthesis drives the global carbon cycle. Net photosynthesis can be quantified at the leaf level by monitoring CO₂ exchange using chamber enclosure systems combined with infrared gas analysers (Long and Bernachi, 2003), and at the ecosystem level using flux towers and eddy covariance techniques (Goulden *et al.*, 1996; Baldocchi, 2008). At the landscape and regional levels, gross photosynthetic CO₂ assimilation, or gross primary productivity (GPP), is inferred using models and algorithms that integrate ground observations with remotely sensed data (e.g. Heinsch *et al.*, 2006; Williams *et al.*, 2009; Jung *et al.*, 2011). Remotely sensed data have been extensively used to infer GPP based on the light use efficiency (LUE) model (Moneith, 1972; Kumar and Moneith, 1981; Zhao *et al.*, 2011). In the LUE model, GPP is proportional to incoming photosynthetically active radiation (PAR), the fraction absorbed by vegetation (*f*APAR), and the LUE at which absorbed radiation is used by photosynthesis:

$$\text{GPP} = \text{PAR } f\text{APAR LUE} \quad (1)$$

Remote sensing has been traditionally used to estimate the first two terms of this equation (see reviews by Hilker *et al.*, 2008; Malenovsky *et al.*, 2009). For example, differences in surface reflectance between the red, blue, and near infrared part of the spectrum have been exploited to derive a wide range of vegetation indices to assess *f*APAR, green biomass, chlorophyll content, or leaf area index (e.g. Rouse *et al.*, 1974; Huete, 1988; Qi *et al.*, 1994; Huete *et al.*, 1997; Daughtry *et al.*, 2000; Haboudane *et al.*, 2002). Typically, vegetation indices show a strong seasonal correlation with GPP in many plant communities (e.g. grasslands, croplands, and deciduous forests), but the correlation breaks down in evergreen plant communities where seasonal changes in GPP are strongly modulated by LUE as well as *f*APAR (Equation 1) (e.g. Sims *et al.*, 2006; Garbulsky *et al.*, 2008). To represent the dynamics of LUE, remote sensing data have been used to classify vegetation into plant functional types (PFTs). Subsequently, global GPP models combine spatially resolved PFT and other remote sensing products [e.g. PAR, *f*APAR, temperature, and vapour pressure deficit (VPD)] with functions and parameters derived from flux tower observations to estimate LUE (Heinsch *et al.*, 2006; Williams *et al.*, 2009; Jung *et al.*, 2011).

Although the LUE approach provides a theoretical basis for constructing and calibrating models, there are no corresponding benchmarks to evaluate model performance at large geographical scales, and model uncertainty remains high (Beer *et al.*, 2010). The situation could be dramatically improved if a new source of data was available that captured the dynamic behaviour of photosynthesis at the relevant scale. Fortunately, photosynthesis generates an optical signal that, in addition to PAR and *f*APAR, is also sensitive to LUE. This signal is chlorophyll *a* fluorescence (ChlF). ChlF are photons of red and far-red light that are emitted by chlorophyll *a* pigments nanoseconds after light absorption. Because photosynthesis and ChlF compete for the same excitation energy, ChlF carries information on LUE.

ChlF has been used for decades to elucidate the organization, function, and acclimation of the photosynthetic apparatus at the subcellular and leaf levels (see seminal reviews by Krause and Weis, 1991; Govindjee, 1995; Lázár, 1999; Maxwell and Johnson, 2000; Baker, 2008). Originally restricted to the laboratory, ChlF measurements made their move to the field with the development of the pulse amplitude-modulated (PAM) technique (an active technique that involves the use of a measuring light and a saturating light pulse), and the subsequent introduction of commercial PAM fluorimeters (Schreiber *et al.*, 1986; Bolhàr-Nordenkamp *et al.*, 1989). PAM fluorometry has facilitated the study of the acclimation of photosynthesis *in situ* and helped clarify the link between ChlF and photosynthetic CO₂ assimilation. Yet, despite the importance and value of PAM fluorometry, for practical reasons the technique has been restricted to the leaf level, and its applicability at the canopy and landscape levels remains unknown. To fill the gap, the field of ChlF has recently seen a new wave of developments that seek to measure ChlF from remote sensing platforms.

The remote sensing technique is based on the passive measurement of solar-induced chlorophyll fluorescence (SIF). The goal is to use the seasonal dynamics in the SIF signal measured from towers, aircrafts, and satellites as a proxy of photosynthesis (Grace *et al.*, 2007; Hilker *et al.*, 2008; Meroni *et al.*, 2009; Rascher *et al.*, 2009). During the last decade, SIF has been successfully measured from tower (Moya *et al.*, 2004; Rossini *et al.*, 2010; Guanter *et al.*, 2013; Drolet *et al.*, 2014), aircraft (Zarco-Tejada *et al.*, 2009, 2012, 2013), and satellite platforms (Guanter *et al.*, 2007; Joiner *et al.*, 2011; Frankenberg *et al.*, 2011; Guanter *et al.*, 2012), the

latter yielding the first global maps of terrestrial ChlF from GOSAT, SCIAMACHY, and GOME-2 (Frankenberg *et al.*, 2011; Joiner *et al.*, 2011, 2013). New space missions, which are in the later stages of development (e.g. NASA OCO-2, GOSAT-2, and ESA Sentinels 4–5), will provide better coverage of SIF data and open up new study possibilities, by using sensors primarily designed to monitor atmospheric chemistry. In turn, the FLEX mission (Moreno *et al.*, 2006), in the latest stage of evaluation by the European Space Agency (ESA), has been specifically designed and optimized to map ChlF at a spatial resolution of 300 m, providing high resolution and global coverage. The amount, quality, and spatiotemporal coverage of SIF data are rapidly increasing. However, we are left with important questions. Are we ready to exploit all the information carried by the SIF signal? Can we export the knowledge obtained from short-term PAM studies to decipher the seasonal dynamics in SIF?

Remote sensing of ChlF takes place at a different spatiotemporal domain and uses a different methodology compared with the majority of ChlF studies in the literature. Amongst others, PAM fluorescence is measured over a broad spectral region whereas SIF is estimated within very narrow spectral bands. PAM fluorescence is not affected by ambient illumination whereas the SIF signal is. Most importantly of all, while we have a good mechanistic understanding of the processes that control the ChlF signal over the short term (from seconds to days), the interplay between the seasonal acclimation of photosynthesis and the ChlF signal remains unknown. Clearly, there is an urgent need to compile and re-examine the underlying theory in the context of the new applications, to identify the open questions, and to establish a roadmap that encourages the needed breakthroughs.

The goal of this review is to introduce and revisit the physical, physiological, and methodological factors that control the ChlF signal in the context of remote sensing applications, and to identify those research questions that remain open. This review is also conceived as a general introduction of ChlF for the broad community involved in the remote sensing of ChlF.

For simplicity, we focus on the leaf level because it is the smallest spatial scale at which fluorescence and photosynthetic CO₂ uptake can be mechanistically linked and measured simultaneously. Up-scaling the signal from the leaf to the scales observed by airborne or spaceborne sensors falls in the domain of canopy–atmosphere radiative transfer, something equally essential to interpret SIF but outside the scope of the present review.

The review is organized in four main sections. In Section 2, we present the basis of photosynthesis and its optical signals to clarify the potential and limitations of optical data, and to introduce the multitude of processes that are embedded in Equation 1. In Section 3 we introduce the biophysical and physiological basis of ChlF from the molecular level to the leaf level and beyond, providing the theoretical and mechanistic knowledge needed to understand the spatiotemporal dynamics of the ChlF signal from the context of remote sensing. In Section 4 we introduce and compare PAM and SIF fluorometry, clarifying the main differences. Finally, in Section

5 we identify and discuss the challenges that remain to be solved, proposing further experimental work. Alternatively, the reader may wish to skip the background theory presented in Sections 2 and 3 and come back to it later on for reference.

2. The regulation of photosynthesis and its optical signals

Photosynthesis involves two main sets of reactions: the light reactions, where electromagnetic energy is absorbed by pigments and converted into chemical energy in the form of ATP and NADPH; and the carbon fixation reactions, where ATP and NADPH are used to produce sugars from atmospheric carbon dioxide. Because the light and carbon reactions exhibit different sensitivities to environmental variables such as light, temperature, or water availability, the production of ATP and NADPH by the light reactions and consumption of these metabolites by the carbon reactions do not always match (Ögren *et al.*, 1984; Huner *et al.*, 1996; Ensminger *et al.*, 2006).

Energy absorbed in excess by the light reactions can damage the photosynthetic machinery (Barber and Andersson, 1992; Demmig-Adams and Adams, 2000; Tyystjärvi, 2013), for example a leaf in a sunny (high energy input) but cold (low energy consumption) environment. Accordingly, plants have evolved a number of regulatory mechanisms to adjust the energy balance between the light and carbon reactions (Walters, 2005; Demmig-Adams and Adams, 2006). The result of this continuous adjustment is that the performance of the light reactions of photosynthesis (visible to optical sensors) tends to emulate that of the carbon reactions. This establishes a link between optical data and GPP that can be implemented to the remote sensing of photosynthesis. Some processes, however, interfere with the relationship, which we discuss below (Fig. 1).

2.1 Light absorption and its regulation

Photosynthesis starts with absorption of light, mainly by chlorophyll molecules. Accordingly, an effective mechanism used by plants to regulate light absorption, or *f*APAR, consists of adjusting the concentration of chlorophyll pigments in the leaf (Fig. 1). The relationship between chlorophyll content and light absorption is non-linear because the increment in light absorption per unit of chlorophyll decreases at high chlorophyll contents (Adams *et al.*, 1990; Gitelson *et al.*, 1998). Net changes in leaf-level chlorophyll are visible over time scales of days (García-Plazaola and Becerril, 2001; Lu *et al.*, 2001). In addition, certain plant species use other mechanisms to modulate photosynthetic light absorption that operate at different temporal scales: leaf movements and leaf angle adjustments (Yu and Berg, 1994; Arena *et al.*, 2008), chloroplast movements (Brugnoli and Björkman, 1992; Sarvikas *et al.*, 2010), changes in surface reflectance mediated by salt bladders (Mooney *et al.*, 1977; Esteban *et al.*, 2013), changes in leaf epicuticular wax properties (Pfundel *et al.*, 2006; Olascoaga *et al.*, 2014), changes in leaf

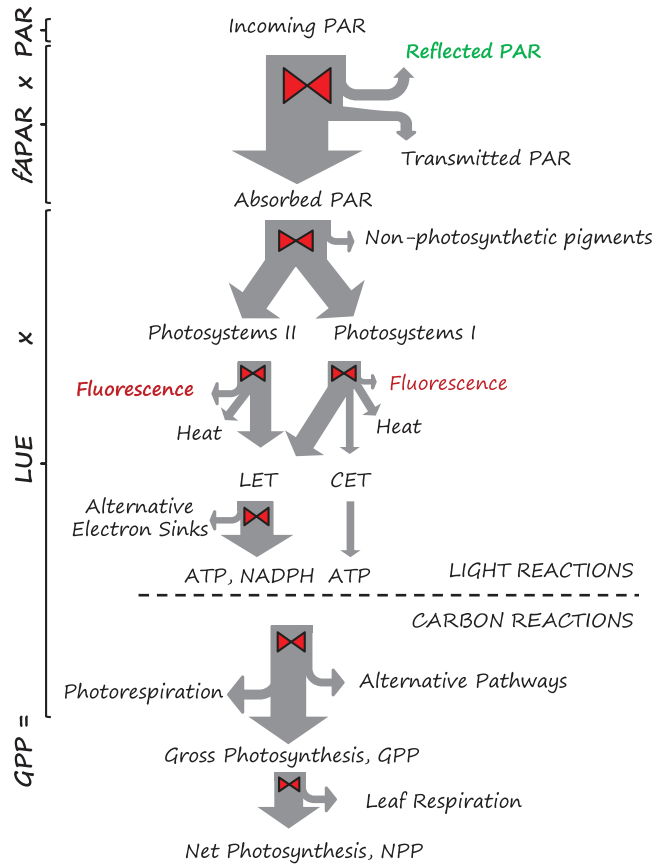


Fig. 1. Photosynthetic energy partitioning at the leaf level and the light use efficiency model ($GPP = PAR \times fAPAR \times LUE$). Red valve symbols indicate the action of regulatory mechanisms which adjust the energy partitioning between pathways (grey arrows). Grey arrows represent the flow of energy. Optical signals available to remote sensing include properties of reflected light (shown in green) and chlorophyll a fluorescence (shown in red). LET, linear electron transport; CET, cyclic electron transport.

surface structures such as pubescence (Ehleringer et al., 1976; Morales et al., 2002; Galmés et al., 2007a), and changes in the concentration of non-photosynthetic pigments such as anthocyanins (Close and Beadle, 2003; Pfündel et al., 2006; Merzlyak et al., 2008) (Fig. 1). The temporal dynamics of these processes need to be considered when interpreting ChlF data because changes in light absorption have a direct impact on ChlF intensity (see Section 3). A special case is that of non-photosynthetic pigments which do not contribute to ChlF or photosynthesis, but increase leaf absorbance (Hlavinka et al., 2013).

2.2 Linear and cyclic electron transport and energy distribution between photosystems

Photosynthetic pigments are bound by proteins to form photosynthetic antenna complexes (Liu et al., 2004) that capture light energy and transfer it to a reaction centre. A reaction centre is a special pigment-protein complex that converts excitation energy to chemical energy. The combination of reaction centre and antenna is termed a photosystem. Higher plants have two types of photosystems: photosystem I (PSI) and photosystem II (PSII), which actually operate in series in the opposite order, that is with electrons being transferred from PSII to PSI (Fig. 2).

After a photon is captured by a chlorophyll molecule in PSII, the excitation energy rapidly reaches the reaction centre chlorophyll, referred to as P680 (a pigment with absorption maximum at 680 nm). Excited P680* rapidly gives an electron to the primary electron acceptor, pheophytin, which in turn reduces the quinone A (Q_A) electron acceptor to yield the first stable charge-separated state P680⁺Q_A⁻. Subsequently, Q_A⁻ passes an electron to quinone B (Q_B) which leaves its binding site when double reduced and protonated by stromal protons.

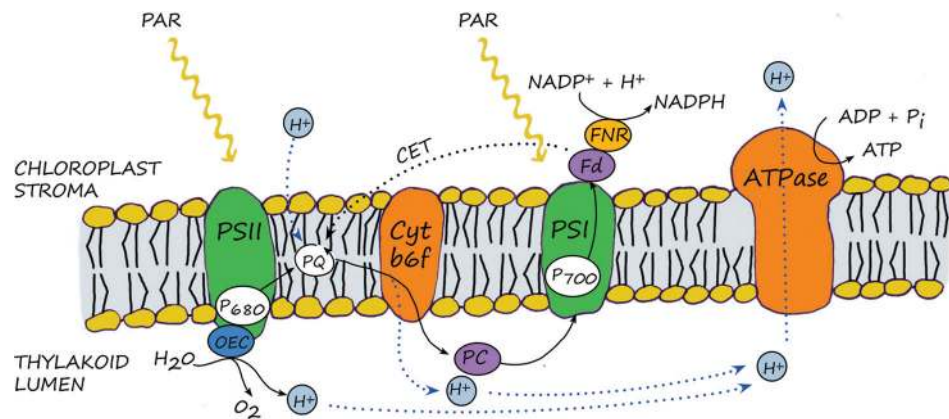


Fig. 2. Schematic representation of the photosynthetic linear electron transport (LET) chain. In LET, excitation energy from absorbed photons in PSII is used to reduce the plastoquinone pool ('PQ') and pump protons from the chloroplast stroma into the thylakoid lumen via the Cyt *b₆f* complex. Energy from absorbed photons in PSII is also used to operate the oxygen-evolving complex (OEC) by which water molecules are split yielding further protons. Simultaneously, the energy from photons absorbed by PSI is used, via ferredoxin (Fd), by ferredoxin-NADPH reductase (FNR) to reduce NADP⁺ to NADPH. The oxidized PSI reaction centre P700⁺ is reduced back to P700 using an electron donated by plastocyanin (PC), originally from PSII. In cyclic electron transport (CET), an electron is passed from Fd back to the PQ pool and again to PSI via PC. This results in pumping of protons to the lumen but no NADPH synthesis. Protons accumulated in the lumen (from either LET or CET) are used by ATP synthase (ATPase) to synthesize ATP.

Protonated Q_B reduces plastoquinone ('PQ', not to be confused with photochemical quenching PQ), which is subsequently reoxidized by the cytochrome b_6f complex (Cyt b_6f), eventually transferring the protons to the thylakoid lumen (Fig. 2). At the donor side, P680⁺ is reduced by tyrosine Z (TyrZ); subsequently TyrZ⁺ takes up an electron from the oxygen-evolving complex (OEC) which is responsible for the splitting of the water molecule and the release of oxygen and protons (Antal *et al.*, 2013). The resulting protons, together with those pumped to the lumen by the Cyt b_6f complex, accumulate in the thylakoid lumen, generating a proton concentration gradient across the thylakoid membrane (Kramer *et al.*, 2004a). This gradient is used by ATP synthase (ATPase) to synthesize ATP. Simultaneously, energy absorbed in PSI is captured by its reaction centre chlorophyll (P700) and used to reduce the electron acceptor ferredoxin (Fd). Oxidized P700⁺ is reduced back to P700 by taking an electron from plastocyanin (PC). From ferredoxin, the electron is passed to NADP⁺ to produce NADPH in a reaction catalysed by ferredoxin-NADPH reductase (FNR). This series of reactions makes up the linear electron transport (LET) (for reviews, see Ort and Yocum, 1996; Antal *et al.*, 2013).

Efficient operation of the LET implies that the populations of PSII and PSI work in series and their reaction centres transfer electrons at approximately similar rates. Although a trivial solution would be to allocate the same relative antenna cross-section area to PSII and PSI, extra flexibility is required. For example, the absorption spectra of PSII and PSI are different due to differences in pigment composition and spectral forms (see Section 3.2), with PSI absorbing light of slightly longer wavelengths (Duysens and Sweers, 1963; Boichenko, 1998; Pfündel, 2009). In particular, PSI has a higher proportion of chlorophyll *a* compared with PSII, with chlorophyll *a/b* ratios of 9 for PSI compared with 2.5 for PSII, in extracted photosystem particles (Ben-Shem *et al.*, 2003; Nield and Barber, 2006). As a result, the probabilities of light absorption by PSII and PSI will change depending on the spectral properties of incoming light, and its temporal and spatial dynamics.

Another factor that calls for extra flexibility in energy partitioning between photosystems is the operation and dynamics of cyclic electron transport (CET) (Joliot and Joliot, 2002; Rumeau *et al.*, 2007). CET translocates electrons around PSI and pumps protons from the chloroplast stroma to the thylakoid lumen (Fig. 2). Because the electron is recycled, CET yields only ATP but no NADPH. Functionally, CET is thought to contribute to the efficient induction of the Calvin–Benson cycle upon illumination of dark-acclimated leaves (Joliot and Joliot, 2002), the regulation of the lumen pH, and thereby modulation of non-photochemical quenching in the photosystems, NPQ (see below) (Kramer *et al.*, 2004a), or the protection of PSI against photoinhibition (Rumeau *et al.*, 2007; Sonoike, 2011). Indeed, CET has been found to be essential for normal carbon fixation in many C_4 plants (Hatch, 1992). To summarize, changes in light quality and CET require flexible mechanisms capable of adjusting the energy partitioning between PSII and PSI (i.e. the relative absorption cross-sections of PSII and PSI, a_{II} and a_I , respectively) (Fig. 1).

At a time scale of minutes, the partitioning of energy between photosystems (a_{II} and a_I) is regulated through a process known as state transitions (Murata, 1969; Haldrup *et al.*, 2001; Tikkanen *et al.*, 2011). Under low light, if leaves are illuminated with light that favours PSII, part of the peripheral antenna complexes of PSII can migrate to serve PSI. This has the effect of balancing the energy input between the photosystems. State transitions are considered to be important only under low light conditions (Rintamäki *et al.*, 1997; Haldrup *et al.*, 2001; Tikkanen *et al.*, 2011), and are therefore of little relevance for remote sensing.

At time scales of days, photosystem stoichiometry and a_{II} and a_I can adjust in response to more sustained changes in light intensity and quality (Anderson *et al.*, 1988; Chow *et al.*, 1990; Durnford and Falkowski, 1997; Pfannschmidt *et al.*, 1999; Haldrup *et al.*, 2001). Light intensity and quality co-vary within a plant canopy, with shaded parts of the canopy or understorey plants receiving light enriched in far-red due to absorption of red light by foliage above. The result is that leaves in shaded environments tend to display higher $a_{II}:a_I$ ratios than more exposed foliage (Anderson *et al.*, 1988; Chow *et al.*, 1990; Rivadossi *et al.*, 1999; Hihara and Sonoike, 2001; Eichelmann *et al.*, 2005; Ballotari *et al.*, 2007). In addition to their functional and energetic role, the spatiotemporal dynamics of a_{II} and a_I have important implications for the interpretation of ChlF because they affect the shape of the fluorescence spectra and the magnitude of the PSI fluorescence contribution (Palombi *et al.*, 2011), as well as the estimation of the LET rate by means of fluorescence (see Section 4.1).

Evidence suggests that CET and energy partitioning between photosystems might be highly dynamic in response to stress and environmental conditions (Martin *et al.*, 1978; Ivanov *et al.*, 2001; Eichelmann *et al.*, 2005; Rumeau *et al.*, 2007). However, the seasonal and spatiotemporal dynamics of these traits remain poorly understood.

2.3 Energy partitioning at the photosystem level

Understanding the processes that control the energy partitioning in PSII is crucial to linking ChlF with photosynthetic CO₂ assimilation. Energy absorbed by pigments of PSII is dissipated by three main pathways: (i) it can be used by photochemistry (by LET); (ii) it can be dissipated non-radiatively as heat; or (iii) it can be re-emitted as a photon of fluorescence. A unique relationship between ChlF and photochemical efficiency cannot be established. This is because non-radiative dissipation of excitation energy is dynamic and under physiological control (see Section 3).

In low light and in the absence of stress, most of the absorbed energy is effectively used by photochemistry, and the excitation lifetime in the antenna of PSII (τ_{PSII}) is short (in the order of hundreds of picoseconds) (Dau, 1994; Lavergne and Trissl, 1995; Gilmore *et al.*, 1995). This results in lowered fluorescence yield. This de-excitation pathway is termed photochemical quenching (PQ) (a term originally coined to denote the quenching of the fluorescence signal but herein used to address the photochemical quenching of excitation

energy). If light intensity increases, the carbon fixation reactions and electron transport chain gradually become light saturated, causing an increase in τ_{PSII} . This results in increased fluorescence yield. The sudden increase in fluorescence yield, observed when subjecting a dark-acclimated leaf to strong illumination, and the subsequent decrease are collectively referred as the Kautsky effect (e.g. Govindjee, 1995). These rapid fluorescence dynamics reflect the rapid reduction and re-oxidation of PSII electron acceptors and their influence on τ_{PSII} (see Section 3.2) (Brody and Rabinovitch, 1957).

Plants are incentivized to keep τ_{PSII} as low as possible, while still permitting photochemical trapping. This acts to minimize the formation of chlorophyll triplet states which might lead to production of singlet oxygen, a hazardous reactive oxygen species (Barber and Andersson, 1992). As a result, plants have evolved a number of regulatory mechanisms that are capable of dissipating the excess quanta as heat (see reviews by Müller *et al.*, 2001; Demmig-Adams and Adams, 2006; Garcia-Plazaola *et al.*, 2012). The operation of these mechanisms results in a decrease in the excitation lifetime in the antenna and subsequently a decrease in the ChlF yield. This type of de-excitation pathway is commonly referred to as non-photochemical quenching (NPQ), after being widely estimated using the fluorescence parameter NPQ (Bilger and Björkman, 1991) (see Section 4.1). However, it should be kept in mind that the regulated thermal dissipation of excitation energy (non-photochemical quenching of excitation energy) and the parameter NPQ (non-photochemical quenching of the fluorescence signal) are not always equivalent; thus, it becomes practical to separate NPQ and NPQ (Porcar-Castell 2011; Garcia-Plazaola *et al.*, 2012). For example, when state transitions reduce the PSII relative absorption cross-section (a_{II}), the fluorescence signal is lowered (and the NPQ parameter increases) because fewer photons are absorbed by PSII (Horton and Hague, 1988), but no change takes place in τ_{PSII} . In the following, we use NPQ and NPQ accordingly.

The regulation of NPQ in PSII involves mechanisms operating at different time scales. In the short term (seconds to hours), two ΔpH -dependent mechanisms appear to regulate thermal energy dissipation in PSII (Müller *et al.*, 2001; Demmig-Adams and Adams, 2006; Garcia-Plazaola *et al.*, 2012). When the electron transport chain saturates, proton accumulation tends to decrease lumen pH (Fig. 2). Subsequently, the PsbS protein acts as a proton sensor activating and de-activating NPQ, while lumen pH regulates the activity of the enzyme violaxanthin de-epoxidase (VDE). The lowering of the pH triggers the de-epoxidation of violaxanthin to zeaxanthin, resulting in amplified NPQ (Demmig-Adams, 1990; Horton *et al.*, 1996; Müller *et al.*, 2001; Jahns and Holzwarth, 2012). This second mechanism operates at time scales of minutes. Together, the protonation of antenna proteins and de-epoxidation of xanthophyll cycle pigments have been traditionally addressed as energy-dependent quenching (qE) (Weis and Berry, 1987; Krause and Weis, 1991; Horton *et al.*, 1996). Recently, another zeaxanthin-dependent (qZ) but ΔpH -independent form of NPQ was found in *Arabidopsis* and suggested also to operate at a time scale of minutes (Nilkens *et al.*, 2010). All these mechanisms modulate NPQ

over the course of the day in response to diurnal fluctuations in light and temperature. These NPQ forms relax in the dark (e.g. overnight) and are accordingly termed flexible or reversible NPQ (Müller *et al.* 2001; Demmig-Adams *et al.*, 2006; Porcar-Castell, 2011).

Over longer time scales (days to weeks) plants face more sustained changes in their environment (e.g. drought or low winter temperatures). At the seasonal scale, the light-harvesting machinery undergoes sustained changes that result in down-regulation of the photochemical quenching capacity (PQ) and up-regulation of the non-photochemical quenching capacity (NPQ) in PSII (Ottander *et al.*, 1991, 1995; Verhoeven *et al.*, 1996; Ensminger *et al.*, 2004; Porcar-Castell *et al.*, 2008a; Porcar-Castell, 2011). These adjustments are termed sustained because they do not recover overnight. The decrease in PQ is associated with the accumulation of damaged/photoinhibited PSII reaction centres. Damage and recovery of reaction centres take place simultaneously (Kok, 1956; Ohad *et al.*, 1984; Greer *et al.*, 1986). Thus photoinhibition becomes apparent when damage occurs faster than recovery and, since the rate constant of photoinhibition is proportional to incoming light intensity (Tyystjärvi and Aro, 1996) while the rate of recovery is temperature dependent (Greer *et al.*, 1986), photoinhibition becomes apparent under strong light and particularly when strong light is combined with low temperatures (Strand and Lundmark, 1987; Ottander *et al.*, 1991; Campbell and Tyystjärvi, 2012; Tyystjärvi, 2013). The term photoinhibition has also been used to denote a decrease in the maximum quantum yield of photochemistry, commonly estimated via the fluorescence parameter F_v/F_m (see Section 4.1). The seasonal decrease in F_v/F_m or 'photoinhibition' has been shown to be caused by reaction centre damage, the presence of sustained NPQ, or a combination of both (Porcar-Castell *et al.*, 2008b). In this review, we use the term photoinhibition to refer to the damage of the reaction centre exclusively.

The increase in sustained NPQ has been associated with the overnight retention of a de-epoxidized xanthophyll cycle, the accumulation of the PsbS protein, the aggregation of light-harvesting complexes (LHCs) (Adams and Demmig-Adams, 1994; Ottander *et al.*, 1995; Verhoeven *et al.*, 1996; Ensminger *et al.*, 2004; Zarter *et al.*, 2006), as well as with changes in the redox properties of the electron acceptors of PSII that promote thermal dissipation in the reaction centre (Krause, 1988; Ivanov *et al.*, 2002, 2008; Matsubara and Chow, 2004); see Verhoeven (2014) for a recent review on sustained forms of NPQ. While the correlation between NPQ (i.e. thermal energy dissipation) and the parameter NPQ (i.e. fluorescence signal quenching) is well understood over the diurnal scale, the correlation between NPQ and NPQ at the seasonal scale remains obscure (Porcar-Castell, 2011), which in turn complicates the interpretation of seasonal time series of ChlF data.

In contrast to PSII, the lifetime of excitation in PSI (τ_{PSI}) does not seem to be affected by photochemical and non-photochemical quenching processes (at least over the short term) possibly because its reaction centre is very efficient in quenching excitation energy in the oxidized state. The result is a relatively low and constant contribution of fluorescence from

PSI to the total signal (Genty *et al.*, 1990b; Pfündel, 1998; Palombi *et al.*, 2011) that can be treated as a constant signal offset (e.g. Porcar-Castell *et al.*, 2006). However, the seasonal dynamics of PSI ChlF remain unknown.

In addition to ChlF, the regulation in energy partitioning at PSII generates another optical signal. The operation of the xanthophyll cycle and the fast regulation of NPQ in PSII are associated with changes in reflectance at ~531 nm (Gamon *et al.*, 1990). This feature is exploited by the photochemical reflectance index (PRI) (Gamon *et al.*, 1992), an index that has been shown to track changes in LUE through its correlation with NPQ (Evain *et al.*, 2004; Nichol *et al.*, 2006). The potential of the PRI as a remote sensing proxy of LUE has been demonstrated (Drolet *et al.*, 2005; Garbulsky *et al.*, 2008). However, the PRI has also been shown to be very sensitive to canopy structure, gap fraction, background, viewing angle, or leaf area index (Barton and North, 2001; Sims *et al.*, 2006; Goerner *et al.*, 2011), complicating the association of PRI and LUE. In addition, although the short-term variation in leaf-level PRI appears indeed to be controlled by NPQ, the seasonal variation in leaf-level PRI seems to be controlled by the slow changes in pigment pools rather than NPQ (Stylinski *et al.*, 2002; Filella *et al.*, 2009; Porcar-Castell *et al.*, 2012). The mechanistic link between the PRI and LUE appears to be highly dependent on scale and remains to be fully elucidated.

2.4 Alternative electron transport sinks and metabolic pathways

ChlF can be used to estimate the rate of LET through PSII (see Section 4.1). However, a number of processes need to be taken into account if we are to infer the rate of gross photosynthetic CO₂ assimilation or the rates of ATP and NADPH production from fluorometric estimates of LET. Because CET produces ATP but no NADPH, fluorometrically estimated LET will decouple from ATP production in the presence of CET (Fig. 1). Additionally, alternative electron transport sinks such as chlororespiration (Nixon, 2000) and the Mehler reaction (Asada, 2000) reduce the overall quantum yield of NADPH, further decoupling LET from NADPH production.

Alternative electron sinks are generally assumed to be small relative to LET, although they play an important functional role and can become significant under certain conditions. For example, the rate of chlororespiration has been shown to increase under high light and high temperature (Diaz *et al.*, 2007) and suggested to contribute to excess energy dissipation in the alpine plant *Ranunculus glacialis* (Laureau *et al.*, 2013). The Mehler reaction, in turn, has been shown to be stimulated under some water stress conditions (Biehler and Fock, 1996; Flexas *et al.*, 1999; Asada 2000). In addition, part of ATP and NADPH produced by the light reactions can be used by alternative metabolic pathways (e.g. nitrate and sulphate reduction in chloroplasts, or emission of plant volatile organic compounds) (Fig. 1). Krivosheeva *et al.* (1996) found that the electron transport rate and photosynthetic CO₂ uptake were decoupled in overwintering Scots pine, suggesting an increase in an alternative electron sink or metabolic pathway. Overall, the action of alternative sinks and metabolic pathways may,

under certain conditions, affect the ability of ChlF to track the dynamics of photosynthetic CO₂ assimilation.

2.5 Carboxylation, oxygenation, and day respiration

The ATP and NADPH generated by the light reactions are utilized by the Calvin–Benson cycle to synthesize sugars by assimilating CO₂ (gross photosynthetic assimilation or A_G) (Fig. 1). Net photosynthetic assimilation (A_N) is the quantity that is measurable by gas exchange systems and relates to ‘true’ or gross photosynthesis (A_G) as:

$$A_N = A_G - PR - R_d \quad (2)$$

where R_d is the rate of mitochondrial day respiration and PR is the rate of photorespiration (Ogren, 1984). In photorespiration, Rubisco catalyses the oxidation (adding O₂) instead of the carboxylation (adding CO₂) of ribulose bisphosphate, with the oxidized product being partially recovered via the emission of CO₂. Because fluorometrically estimated LET (see Section 4.1) relates to A_G rather than A_N , the magnitude of photorespiration and mitochondrial respiration needs to be taken into account when comparing gas exchange and ChlF measurements.

In C₄ plants (e.g. maize and sorghum), photorespiration is almost fully suppressed, and fluorometrically estimated LET correlates well with net photosynthesis (e.g. Genty *et al.*, 1990a; Krall and Edwards, 1992). In C₃ plants, which include practically all tree species and the majority of higher plants, the energy flow going to photorespiration is considerable and variable. Flexas and Medrano (2002) showed that for a pool of different species the quantum yield of photorespiration increased in response to mild water stress, from 18% to 22%, while the quantum yield of photochemistry stayed approximately constant. Thus, processes such as photorespiration may undermine the capacity of ChlF to track plant stress under certain conditions.

The temperature sensitivity of mitochondrial respiration may vary seasonally, diurnally, or within a plant, in response to variations in maintenance respiration (Atkin *et al.*, 2005). For example, R_d has been found to increase from 15% of A_N to as much as 50% in response to drought stress (Flexas *et al.*, 2005; Galmés *et al.*, 2007b), and a similar temporal pattern has been observed in boreal Scots pine foliage, with higher R_d values during spring recovery of photosynthesis compared with summer (Kolari *et al.*, 2007). In summary, CET, alternative electron sinks, photorespiration, and mitochondrial respiration can all decouple optical data such as ChlF from net photosynthetic CO₂ assimilation.

3. Physical and physiological controls of chlorophyll a fluorescence across space and time

In this section we describe how the intensity, spectrum, and dynamics of the ChlF signal *in vivo* are controlled by a number of scale-dependent physical and physiological factors (including photosynthesis).

Fluorescence is radiative loss of the energy of absorbed photons. Because part of the energy of the absorbed photon is lost as heat, the energy of the emitted photon is usually lower (longer wavelength) than that of the absorbed photon, a phenomenon known as Stokes shift. The quantum yield of fluorescence emission depends on both the properties of the fluorescing chromophore and its surroundings. Chlorophylls in ether are highly fluorescent, with quantum yields of 30% and 15% for chlorophyll *a* and chlorophyll *b*, respectively (Latimer *et al.*, 1956; Barber *et al.*, 1989). Carotenoids, in turn, yield very little fluorescence (Gillbro and Cogdell, 1989). In contrast, the quantum yield of ChlF *in vivo* does not exceed 10%, with typical values under steady-state illumination of 0.5–3% (Latimer *et al.*, 1956; Brody and Rabinovitch, 1957; Krause and Weis, 1991). This drastic decrease is due to the photochemical and non-photochemical quenching of excitation energy in the photosynthetic antennae, making ChlF such a valuable tool for assessing photosynthesis.

3.1 Chlorophyll fluorescence at the molecular level

Energy levels of atomic and molecular orbitals are quantized, but molecules present wide, continuous absorption and emission (fluorescence) spectra because vibrational energies of the molecule are superimposed on each electronic energy level.

A photon can be absorbed if its energy equals the difference in the sum of electronic, vibrational, and rotational energies between an excited-state orbital and the ground-state orbital. Vibrational energy is vibration of atoms about their equilibrium positions in the molecule. Because photosynthetic pigments *in vivo* are tightly packed into a protein matrix, the vibration of the chemical bonds in the pigment–protein complex add additional variability to the absorption and emission spectra of pigments *in vivo*. This phenomenon, known as inhomogeneous broadening, explains why photosynthetic pigments display different spectral forms *in vivo* (Vassiliev and Bruce, 2008) but not when isolated. Rotational energy is associated with the rotation of the molecule around its axis, which is negligible in the solid state.

In Fig. 3 we portray an idealized representation of the energy levels and possible energy dissipation pathways for a chlorophyll *a* molecule embedded in a photosynthetic antenna. Chlorophyll *a* absorbs photons of blue and red light very efficiently. Absorption of a blue photon raises an electron from the ground-state orbital (S_0) to an orbital of a high excited state. Part of the energy is transformed to molecular vibrations and rapidly dissipated (Jennings *et al.*, 2003). Subsequently, because vibrational energy levels from higher excited states overlap with those of the S_1 level, electrons originally in an orbital of a higher excited state can switch to

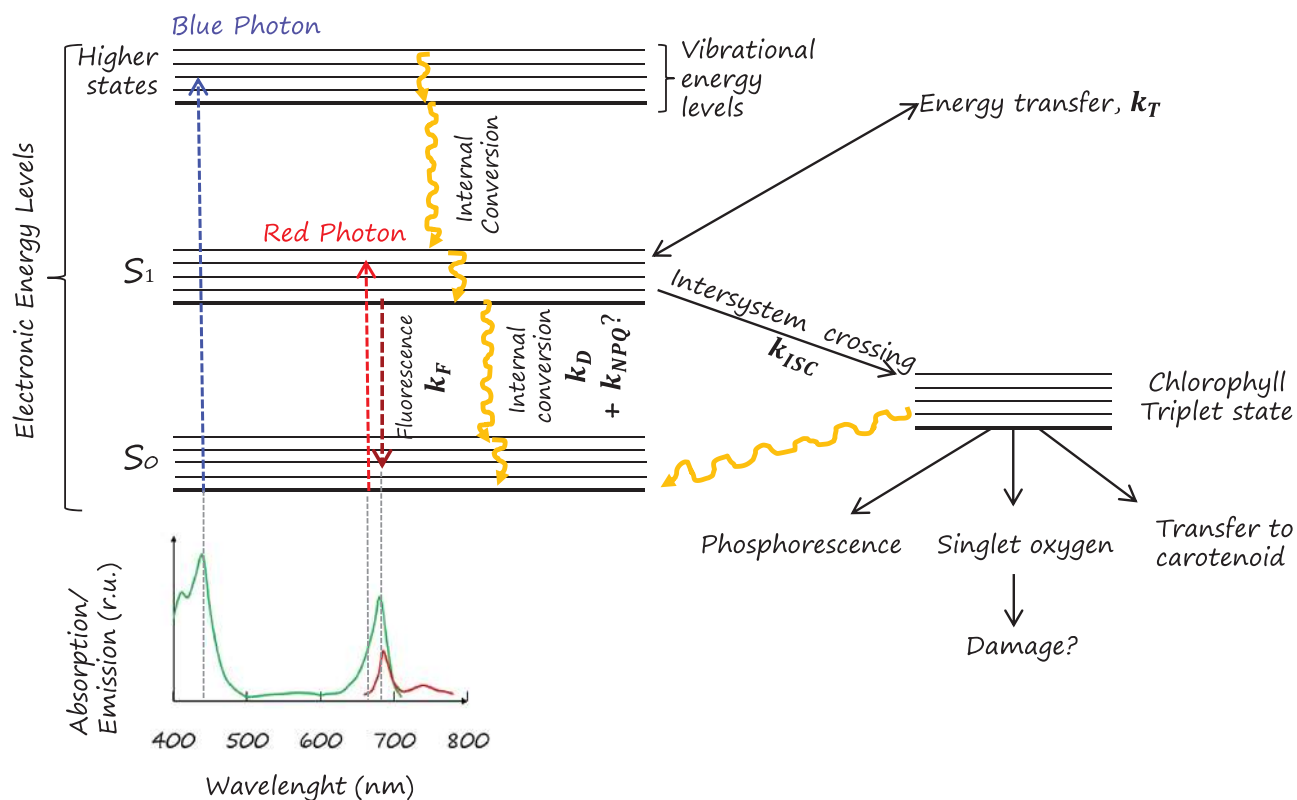


Fig. 3. Idealized Jablonski diagram illustrating the energy partitioning of absorbed photons in a chlorophyll *a* molecule. Upon absorption of a photon of blue light, an electron from the ground state is raised to a higher energy state. The energy is rapidly dissipated non-radiatively as heat mainly by internal conversion, and the electron rapidly relaxes to the first excited state (S_1). In contrast, absorption of a red photon produces the S_1 state directly. It is from S_1 that photosynthetic energy partitioning starts. The electron can relax to the ground state via emission of a chlorophyll fluorescence photon (with an associated rate constant k_F), via non-radiative thermal dissipation, by means of either constitutive (k_D) or physiologically regulated mechanisms (k_{NPQ}), via energy transfer to another pigment or to the reaction centre chlorophyll (k_T), or via intersystem crossing to form a chlorophyll triplet state (k_{isc}). In turn, triplet states can be deactivated via phosphorescence, reaction with oxygen to form singlet oxygen, or by transfer to a carotenoid.

the vibrational manifold of the S_1 level, and the vibrational energy is again rapidly dissipated. The phenomenon is called internal conversion and it populates the first excited state of a chlorophyll molecule in hundreds of femtoseconds to 10 picoseconds after absorption of a photon (Dau and Sauer, 1996; Jennings *et al.*, 2003; Clegg, 2004). A photon of red light produces the S_1 state directly. It is important to note that the difference in energy between a photon of red light and a photon of shorter wavelengths (higher energy) is virtually always dissipated as heat; for this reason, it is more practical to express photosynthetic efficiencies on a quantum rather than an energy basis.

Energy partitioning takes place from S_1 . Excitations in the S_1 state live a thousand times longer compared with those in the higher energy states, and therefore other processes compete with internal conversion for dissipation of excitation from S_1 to S_0 (Fig. 3). In addition to internal conversion, the excitation energy can be transferred to another pigment. Excitation energy can also be lost via emission of a fluorescence photon. Furthermore, a change in the spin state can occur and produce a chlorophyll triplet state, a process called intersystem crossing (Clegg, 2004). The triplet state is long lived and relaxes either by reacting with oxygen to produce reactive singlet oxygen (Tyystjärvi, 2004), by transfer of energy to a carotenoid or other prenyllipid, by internal conversion to the ground state, or by emission of a phosphorescence photon (Hoff, 1986). Finally, the rate constant of thermal dissipation of S_1 states appears to be under physiological control at the photosystem level, at least in PSII. This regulated component of thermal dissipation corresponds to the NPQ mechanism described in Section 2.3. There is no consensus on whether NPQ is enhancement of internal conversion (e.g. by a mechanism that brings chlorophyll *a* molecules close to each other), enhancement of transfer of energy from chlorophyll *a* to zeaxanthin (followed by internal conversion producing the ground state of the carotenoid), or both (Owens *et al.*, 1992; Holt *et al.*, 2005; van Grondelle and Novoderzhkin, 2006). We choose to introduce NPQ already at the molecular level for consistency although NPQ would be meaningful at the level of photosystem. Macroscopically, constitutive or basal thermal dissipation (D) is defined as the minimum thermal dissipation rate obtained in the absence of stress and down-regulation. Any increase in thermal dissipation on top of that is attributed to NPQ.

The quantum efficiency of a process (i) that competes for excitation energy with n other processes, and where k_i is the first-order rate constant of the i th process, can be expressed as (Govindjee, 2004):

$$\Phi_i = \frac{k_i}{\sum_{i=0}^{i=n} k_i} \quad (3)$$

Accordingly, the quantum yield of fluorescence at the molecular level (Fig. 3) can be expressed as:

$$\Phi F_{\text{Chla}} = \frac{k_F}{k_D + k_F + k_{\text{ISC}} + k_{\text{PB}} + k_T (+k_{\text{NPQ}})} \quad (4)$$

where k_D is assumed to be the first-order rate constant associated with the process of internal conversion, k_F is the rate constant of fluorescence emission, k_{ISC} is the rate constant of intersystem crossing leading to chlorophyll triplet states, k_{PB} the rate constant of photobleaching or destruction of the chlorophyll molecule, k_T the rate constant of energy transfer to a neighbouring pigment molecule, and k_{NPQ} the rate constant of regulated thermal energy dissipation (NPQ). All reactions are of the first order and therefore the rate constants are expressed as 1/time unit (s^{-1}). The lifetime of excitation is the inverse of the sum of all rate constants ($\tau = 1/\sum k_i$). The rate constant of fluorescence (k_F) depends on the properties of the chlorophyll molecule and is assumed to remain constant in physiological processes (Butler and Kitajima, 1975; Clegg, 2004). The rate constant k_D is invariable by definition (i.e. any variation is embedded in k_{NPQ}). The rate constants k_{ISC} and k_{PB} are orders of magnitude smaller than k_D or k_F (Clegg, 2004; Santabarbara *et al.*, 2007) and, since they are not relevant for energy partitioning, we hereafter consider them as part of k_D . Finally, the rate constant of energy transfer between pigments k_T includes two main mechanisms: coherent energy transfer, and Förster or fluorescence resonance energy transfer (FRET) (Nedbal and Szöcs, 1986; Clegg, 2004; Engel *et al.*, 2007; Novoderzhkin and van Grondelle, 2010). In general, energy transfer between closely coupled pigments within a single antenna protein can be described as movement of delocalized excitons (coherent energy transfer), whereas energy transfer between pigment–protein complexes requires consideration of localized excited states (Förster energy transfer) (Novoderzhkin and van Grondelle, 2010).

3.2 Chlorophyll fluorescence at the photosystem and thylakoid membrane level

Interpreting ChlF dynamics at the level of thylakoid membrane requires the use of a number of assumptions the validity of which depends on the temporal context of the application. The assumptions listed below are generally accepted when interpreting slow ChlF dynamics (seconds to minutes), but they are too simplified to interpret fast fluorescence kinetics successfully (picosecond to second range) (e.g. Lazár, 1999; Zhu *et al.*, 2005; Stirbet, 2013). Similarly, some of the assumptions may be equally challenged at the seasonal scale, something that will be addressed in this section.

Assumption (A): single pool model. Photosynthetic antennae have evolved to collect photons and effectively deliver the energy to a reaction centre (Fig. 4). Efficient energy transfer from pigment to pigment and to the reaction centre is very rapid (time constants 10 fs to 10 ps) (Clegg, 2004; Engel *et al.*, 2007; Novoderzhkin and van Grondelle, 2010) in comparison with the 200–500 ps required for stable charge separation (Roelofs *et al.*, 1992; Vassiliev and Bruce, 2008). A large body of evidence supports the idea of rapid excitation equilibration in the antennae of PSII and PSI (Schatz *et al.*, 1988; Croce *et al.*, 1996; Dau and Sauer, 1996; Andrizhievskaya *et al.*, 2004; Miloslavina *et al.*, 2006). If we assume rapid excitation equilibration, the antenna, core, and reaction centre can be treated as a single pigment pool

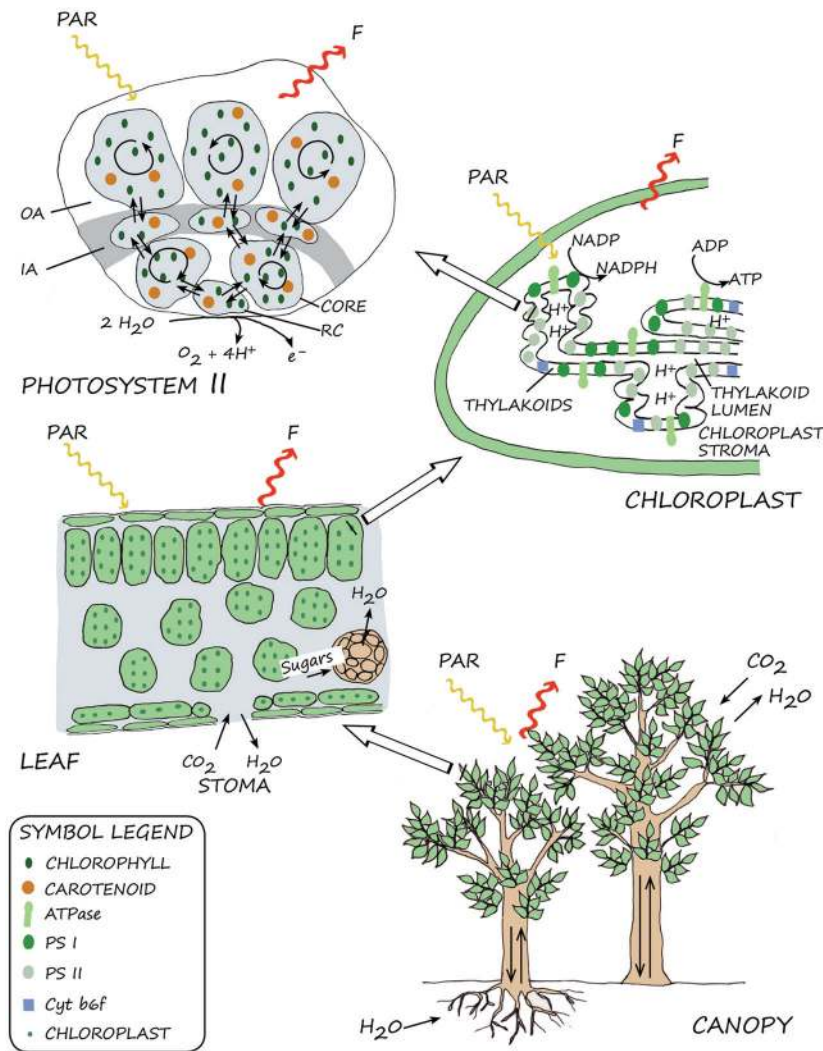


Fig. 4. Chlorophyll a fluorescence and photosynthesis from the photosystem to the canopy level. (A) At the photosystem level, excitation energy is transferred (black arrows) between pigments from the outer antenna (OA), inner antenna (IA), core, and reaction centre (RC) where it can be used to drive photochemistry. A variable part of this excitation energy is lost as heat or re-emitted as chlorophyll fluorescence (F). At the photosystem level, the quantum yield of fluorescence largely depends on the capacity of the above photochemical and non-photochemical processes, whereas the fluorescence spectral properties largely depend on chlorophyll–protein conformation and antenna structure. (B) At the chloroplast level, populations of photosystem II (PSII) and photosystem I (PSI) cooperate for light absorption and together contribute to the resulting fluorescence signal, with quantum yield and spectral properties differing between photosystems. (C) At the leaf level, cells and their chloroplasts are arranged to optimize light absorption within the leaf. This distribution results in important wavelength-dependent light gradients within the leaf and wavelength-dependent reabsorption of fluorescence. (D) At the canopy level, strong vertical gradients in light quality and intensity produce gradients in photosystem size, PSII:PSI stoichiometry, thylakoid organization, leaf morphology, and leaf pigment concentrations. The chlorophyll fluorescence signal and its relationship to photosynthesis increase in complexity with increasing scale.

(Dau, 1994; Lavergne and Trissl, 1995). This greatly simplifies the analysis of ChlF data (Fig. 4).

Assumption (B): no spillover. We consider that the populations of PSII and PSI are energetically isolated and that the rate of transfer of excitations from PSII to PSI (Kitajima and Butler, 1975b; Trissl and Wilhelm, 1993; Tan *et al.*, 1998), known as spillover, can be assumed to be insignificant at ambient temperature.

Assumption (C): no thermal dissipation by closed reaction centres. We consider that thermal energy dissipation by closed reaction centres (with P680⁺) is only relevant when analysing ChlF data that have been excited using intense laser sources, in which the time elapsed between absorption

of two consecutive photons by PSII is shorter than the lifetime of P680⁺ (Shinkarev and Govindjee, 1993).

Assumption (D): no quenching by oxidized plastoquinone. Excitation quenching by oxidized plastoquinone is relatively small (Vernotte *et al.*, 1979), with a rate constant that reaches $0.15(k_f + k_D)$ when at its maximum (Zhu *et al.*, 2005) (i.e. equivalent to $NPQ=0.15$).

Assumption (E): perfect connectivity/lake model. The main link between ChlF dynamics and photosynthesis dynamics originates at the level of PSII, via the photochemical reaction. The photochemical reaction is defined as the stable charge separation including reduction of the Q_A electron acceptor and advancement of the Kok

cycle (Britt, 1996). Photochemistry can only take place in reaction centres that are open and functional (i.e. are associated with an oxidized Q_A and fully operational). We define the fraction of open and functional reaction centres q , which ranges from zero (when all reaction centres are closed) to one (when reaction centres are open and functional). Accordingly, ChlF will increase with decreasing q but the relationship depends on the degree to which excitation can move from closed to open PSII centres, a phenomenon known as connectivity (Joliot and Joliot, 1964; Havaux *et al.*, 1991; Lavergne and Trissl, 1995; Kramer *et al.*, 2004b) and recently reviewed by Stirbet (2013).

If k_{CH} is the rate constant of the excitation energy transfer between two connected PSII units (see Appendix 1), then three different types of connectivity models can be defined (Dau, 1994; Lavergne and Trissl, 1995; Stirbet, 2013): a separate units or puddle model, where $k_{CH}=0$; a lake model, where all photosystems share excitation from a single pool and $k_{CH}=\infty$; and models with finite connectivity ($0 < k_{CH} < \infty$). Importantly, except for the special cases when $q=0$ (termed maximal fluorescence F_M), or when $q=1$ (termed minimal fluorescence F_0) (Kitajima and Butler, 1975a; Dau, 1994; Lavergne and Trissl, 1995), connectivity will interact with the relationship between ChlF and q . Accordingly, it is important to select a model that approximates the level of connectivity in the sample. Two extreme approaches have been used to estimate q : (i) the qP parameter (Schreiber *et al.*, 1986; van Kooten and Snel, 1990) based on a separate units model; and (ii) the qL parameter (Kramer *et al.*, 2004b; Baker, 2008; Porcar-Castell, 2011) based on a lake model, where $qL=q$ for a system with perfect connectivity, and $qP=q$ for a system with no connectivity at all (Appendix 1), with intermediate qL and qP performances depending on connectivity (see Fig. 5 and text below).

The bulk of experimental data favours the view that PSII units are connected to some extent (Joliot and Joliot, 1964; Dau *et al.*, 1994; Lavergne and Trissl, 1995; Kramer *et al.*, 2004b; Tyystjärvi *et al.*, 2009) although some recent data favour a model of isolated centres (Oja and Laisk, 2012). Overall, the lake model has been described as a reasonable assumption to study the slow ChlF dynamics dealt with here (Kramer *et al.*, 2004b; Baker, 2008). These conclusions need to be re-assessed at the seasonal scale for three reasons: (i) The effect of connectivity (excitation transfer from closed to open centres) could expand to photoinhibited reaction centres (excitation transfer from damaged to functional centres); (ii) connectivity might undergo changes at the seasonal scale; and (iii) connectivity effects have been analysed in the absence of or under constant and relatively low levels of NPQ, but the interplay between connectivity and NPQ remains unknown.

Lavergne and Trissl (1995) derived equations for examining the impact of different connectivity models on fluorescence yield as a function of the fraction of open reaction centres (q). Applying those equations (Appendix 1), we evaluated the impact of NPQ on connectivity and the interplay between connectivity, NPQ, qL , and qP (Fig. 5). The analysis demonstrates that connectivity has very little effect

on ChlF in the presence of NPQ (Fig. 5A–D). Therefore, the impact of connectivity on seasonal changes of ChlF obtained under natural illumination (as in the remote sensing of SIF) is probably small. In contrast, selecting a proper connectivity model remains important even in the presence of NPQ if q needs to be estimated. In a nutshell, overestimation of q by qP increases with connectivity, and underestimation of q by qL decreases with connectivity (Fig. 5E–L). If we use the lake model assumption to interpret ChlF data, a seasonal decrease in connectivity would translate into underestimation of q and consequently to underestimation of ΦP (Equation 9). A decrease in connectivity would also decrease the overall probability of photochemical excitation trapping by reaction centres. Accordingly, the effects of connectivity on ChlF and on the ‘true’ ΦP tend to cancel out (Weis and Berry, 1987), and therefore fluorometric estimates of ΦP are expected to be good proxies of ‘true’ ΦP irrespective of connectivity issues. Keeping this in mind, we use the lake model assumption and the parameter qL as a proxy of q to demonstrate the derivation of the quantum yield of photochemistry in PSII (see Section 4.1 for application to PAM fluorometry).

The effective rate constant of photochemistry (k_p) depends on the maximum intrinsic rate constant of photochemistry (k_{PSII}) proportionally to qL as:

$$k_p = qLk_{PSII} \quad (5)$$

Subsequently, the fluorescence flux of PSII (F_{II}) ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) emanating from a sample with area (m^2) that contains a large population of both PSII and PSI units can be expressed as a function of the quantum yield of fluorescence emission (ΦF_{II}):

$$F_{II} = IAa_{II} \Phi F_{II} \quad (6)$$

where I is the incident flux of photosynthetic active radiation ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$), A is the absorptance coefficient, and a_{II} the relative absorption cross-section area of the PSII population in the sample. Applying Equation 3, ΦF_{II} can be expressed as:

$$\Phi F_{II} = \frac{k_F}{k_D + k_F + k_{NPQ} + qLk_{PSII}} \quad (7)$$

Equations 6 and 7 introduce the link between ChlF and photochemistry at the level of PSII. It should be clarified that these equations (and the rate constants therein) are only meaningful when applied to large populations of photosystems. Because both k_{NPQ} and qL vary in response to the acclimation of photosynthesis, a direct link between ChlF and photochemistry cannot be readily established with Equation 7 alone. An additional fluorescence measurement in a different state is needed.

Estimation of the quantum yield of photochemistry of PSII (ΦP) is accomplished by measuring ChlF in the presence ($0 < q < 1$) and in the absence of photochemistry ($q=0$). This has been traditionally accomplished using herbicides such as DCMU (e.g. Horton and Hague, 1988), liquid nitrogen temperature (Björkman and Demmig, 1987), and more

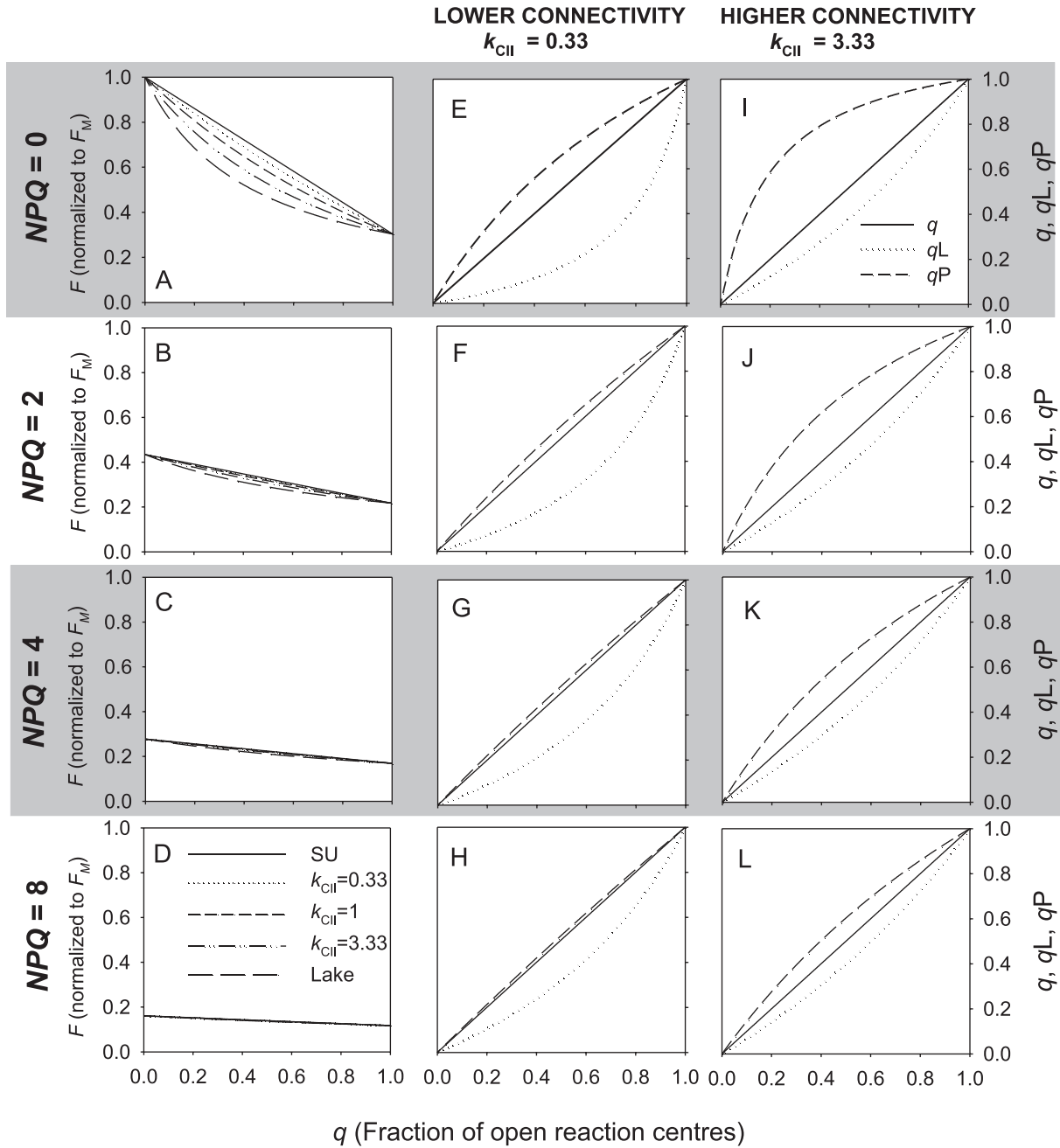


Fig. 5. Theoretical analysis of the interaction between photosystem II connectivity, the fraction of open reaction centres q , NPQ, and the parameters qL and qP . (A–D) The effect of increasing levels of NPQ on the relationship between F and q at different levels of connectivity, where k_{CII} is the rate constant of energy transfer between photosystems, ranging from zero, for the case of separate units (SU), to infinity, for a lake model. (E–L) Theoretical relationship between q and the fluorometric estimates of q based on a separate units assumption (qP) or on a lake model (qL) under different levels of NPQ and under two different connectivity scenarios. Derived using parameters and equations after Lavigne and Trissl (1995); see Appendix 1 for details.

commonly saturating light pulses (Schreiber *et al.*, 1986; see Section 4.1). The result is an increase in F_{II} to a maximal level (F_{IIm}). Where,

$$F_{IIm} = IAa_{II} \frac{k_F}{k_D + k_F + k_{NPQ}} \quad (8)$$

Since the sum of the quantum yields of all processes competing for excitation energy equals one, the quantum yield of photochemistry in PSII (ΦP) can be expressed in fluorescence terms as:

$$\begin{aligned} \Phi P &= \frac{qLk_{PSII}}{k_D + k_F + k_{NPQ} + qLk_{PSII}} \\ &= 1 - \frac{k_D + k_F + k_{NPQ}}{k_D + k_F + k_{NPQ} + qLk_{PSII}} = \\ &= 1 - \frac{k_F}{\frac{k_D + k_F + k_{NPQ} + qLk_{PSII}}{k_F}} = 1 - \frac{F_{II}}{F_{IIm}} \end{aligned} \quad (9)$$

which corresponds to the parameter derived by Genty *et al.*, (1989) at the level of PSII, and becomes ΦP_{\max} (the widely used parameter F_V/F_M ; see Section 4.1) when $qL=1$ and $k_{\text{NPQ}}=0$.

Because the rate constants k_D and k_F remain constant, variations in ΦP can be expressed as a function of photochemical and non-photochemical processes, with $k_D+k_F=1$. This simplification gives rise to the photochemical (PQ) and non-photochemical quenching (NPQ) parameters, where

$$\text{PQ} = qL \frac{k_{\text{PSII}}}{k_D + k_F} \quad \text{and} \quad \text{NPQ} = \frac{k_{\text{NPQ}}}{k_D + k_F} \quad (\text{Laisk } et al., 1997;$$

Porcar-Castell, 2011), and the quantum yield of photochemistry can be expressed as:

$$\Phi P = \frac{\text{PQ}}{1 + \text{NPQ} + \text{PQ}} \quad (10)$$

Note that the two forms of photochemical quenching parameter, PQ and qL , carry exactly the same information and can be used interchangeably depending on the application. For example, PQ facilitates the comparative analysis with NPQ because it has the same relative range of variation, whereas qL gives a more visual picture of the degree of reaction centre openness.

In contrast to PSII, the fluorescence yield of PSI is generally low and remains constant under illumination. Chlorophyll cations such as P700⁺ and P680⁺ are known to be very efficient in dissipating excitation energy as heat. However, in contrast to P680⁺, the lifetime of P700⁺ is longer and P700⁺ operates as a very effective quencher of excitation energy in PSI (Dau, 1994; Lavergne and Trissl, 1995). The fluorescence spectral properties of PSII and PSI are also different (Fig. 6). At room temperature, ChlF from plant PSI presents a shoulder at 690 nm and a peak at 730 nm (Croce *et al.*, 1996), whereas fluorescence from PSII peaks at ~685 nm and presents vibrational satellite bands in wavelengths >700 nm (Govindjee, 1995) overlapping with PSI fluorescence (Franck *et al.*, 2002; Fig. 6). As a result of differences in yield and spectral properties between photosystems, PSI fluorescence has been found to contribute between 0 and 50% of total fluorescence depending on the method, measuring conditions, species, and especially the spectral region, with the contribution of PSI being insignificant in the red region and maximum in the near infrared (Fig. 6) (Genty *et al.*, 1990b; Dau, 1994; Pfündel, 1998; Agati *et al.*, 2000; Peterson *et al.*, 2001; Franck *et al.*, 2002; Palombi *et al.*, 2011; Pfündel *et al.*, 2013).

Analogously to PSII, we express the fluorescence flux emanating from a population of PSI units as:

$$F_I = IAa_I \frac{k_F}{k_D + k_F (+k_{\text{NPQI}}) + qk_{\text{PSI}} + k_{\text{RCI}}} \quad (11)$$

where a_I is the relative absorption cross-section area of PSI in the sample, q is the fraction of open PSI centres, k_{PSI} is the intrinsic rate constant of PSI photochemistry, and k_{RCI} is the rate constant of quenching by P700⁺. Finally, k_{NPQI} is the rate

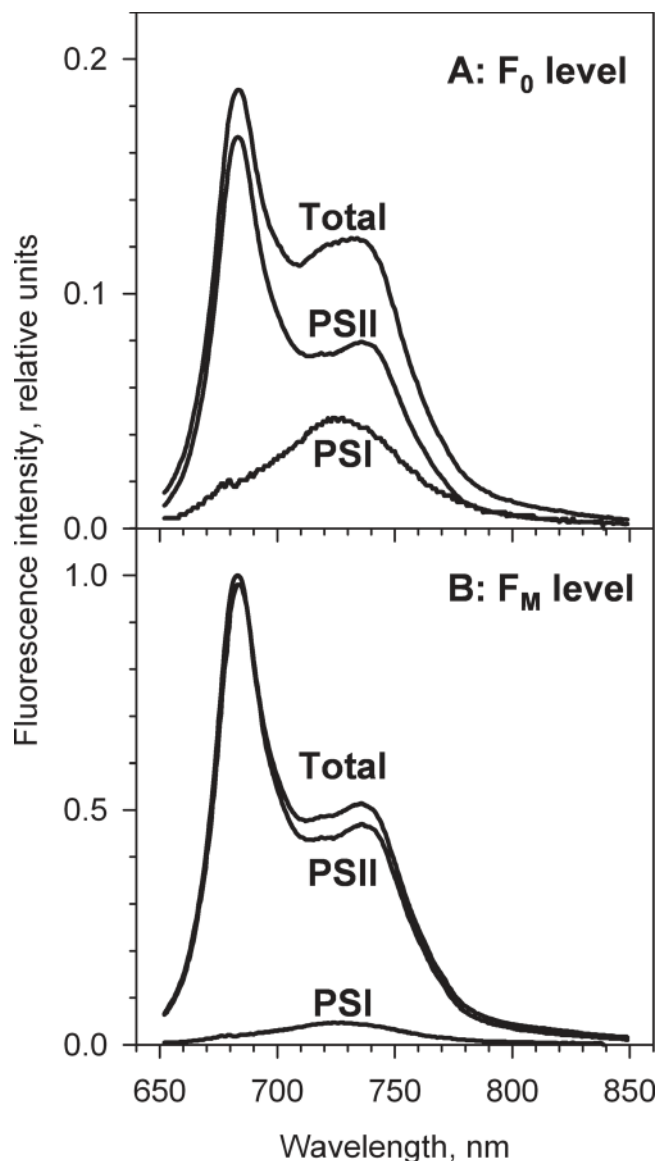


Fig. 6. The relative spectral contributions of PSI and PSII fluorescence to the total emission spectrum in barley leaves at the F_0 (A) and F_M (B) levels. Fluorescence is normalized to the peak in F_M . Redrawn after Franck F, Juneau P, Popovic R. 2002. Resolution of the photosystem I and photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. *Biochimica et Biophysica Acta* 1556, 239–246. Copyright Elsevier 2002.

constant of regulated thermal energy dissipation in PSI, the significance of which remains to be elucidated (see Section 5).

3.3 Chlorophyll fluorescence at the leaf level and beyond

A new set of factors and phenomena need to be considered when linking ChlF data and photosynthetic CO_2 assimilation at the leaf level and beyond (Fig. 4). The efficient absorption of blue and red light by chlorophyll within a leaf or by leaves within a plant canopy plays two important roles when scaling from the photosystem to the leaf level and beyond: (i) wavelength-dependent light penetration; and (ii) wavelength-dependent fluorescence reabsorption.

First, red light penetrates deeper into the leaf and is more scattered than blue light (Agati, 1998; Buschmann and Lichtenthaler, 1998; Vogelmann and Evans, 2002), whereas green light may have an optical path up to five times longer than that of red or blue light due to weaker absorption and higher scattering (Vogelmann, 1993; Rappaport *et al.*, 2007). Wavelength-dependent scattering and absorption within a leaf or a plant canopy generates important gradients in light quality and intensity which translate into similar gradients in thylakoid composition, stoichiometry, and physiological state. For example, the number of PSII reaction centres is known to increase with irradiance (Anderson *et al.*, 1988) or in response to far-red light (Chow *et al.*, 1990), whereas the antenna sizes of the photosystem (Melis, 1991; Anderson *et al.*, 1988; Ballottari *et al.*, 2007), as well as the absorption cross-section of PSII relative to PSI (Eichelmann *et al.*, 2005; Walters, 2005) both increase in response to shade. On the other hand, xanthophyll pigment pools and NPQ capacity are larger in sun-exposed compared with shaded foliage (Demmig-Adams, 1998; Niinemets *et al.*, 2003; Porcar-Castell *et al.*, 2008a). In addition, chloroplasts or leaves exposed to high light conditions tend to have lower photochemical efficiencies and larger NPQ compared with more shaded chloroplasts or leaves.

Secondly, because the spectra of ChlF emission overlaps with that of chlorophyll absorption, red fluorescence photons (F_R) can be reabsorbed by chlorophyll itself within the leaf or inside a plant canopy (Brody and Brody, 1962; Govindjee and Yang, 1966; Gitelson *et al.*, 1998). Reabsorption of F_R within the leaf can be as high as 90% (Gitelson *et al.*, 1998).

The overall result of the above two phenomena is that the biological footprint of a fluorescence measurement depends on the spectral properties of both the excitation light and the wavelengths across which fluorescence is retrieved (Fig. 7). The F_R signal (detected in the red region) is enriched in photosystems close to the leaf surface or leaves from the top of the canopy, whereas the F_{FR} signal (detected in the far-red)

may have a stronger contribution from a deeper leaf or canopy layer, especially when the excitation light penetrates deep into the leaf or the canopy (Peterson *et al.*, 2001; Rappaport *et al.*, 2007; Pfündel, 2009). These differences are particularly relevant to the interpretation of SIF data where the signal can be obtained in different spectral regions that will consequently carry information from different layers of the leaf or the canopy (see Section 4.2).

Although Equations 6 and 7 provide the basis for linking ChlF and photochemistry, the effects of leaf and canopy structure also affect the ChlF signal and need consideration. A quantitative treatment of the impact of structure requires the use of leaf and canopy radiative transfer models. Here we use a simplified expression to introduce the main physical and physiological controls behind leaf-level ChlF (for similar formulations, see also Agati *et al.*, 1995; Dau and Sauer, 1996; Franck *et al.*, 2002; Pedrós *et al.*, 2008; Palombi *et al.*, 2011). The intensity and spectral properties of the chlorophyll fluorescence signal emanating from a leaf F can be represented (in $\mu\text{mol photons m}^{-2} \text{s}^{-1} \text{nm}^{-1}$) as:

$$F \left[I(\lambda_{\text{ex}}), A(\lambda_{\text{ex}}), a_{\text{II}}(\lambda_{\text{ex}}), a_{\text{I}}(\lambda_{\text{ex}}), F_{\text{PSII}}(\lambda_{\text{em}}), F_{\text{PSI}}(\lambda_{\text{em}}), \text{NPQ}, qL, p_r(\lambda_{\text{em}}, \text{Chl}) \right] = I(\lambda_{\text{ex}}) A(\lambda_{\text{ex}}) \left[a_{\text{II}}(\lambda_{\text{ex}}) \Phi_{\text{FII}}(k_{\text{NPQ}}, qL) F_{\text{PSII}}(\lambda_{\text{em}}) + a_{\text{I}}(\lambda_{\text{ex}}) \Phi_{\text{FI}} F_{\text{PSI}}(\lambda_{\text{em}}) \right] \left[1 - p_r(\lambda_{\text{em}}, \text{Chl}) \right] \quad (12)$$

where $I(\lambda_{\text{ex}})$ and $A(\lambda_{\text{ex}})$ are the irradiance flux at the leaf surface and the leaf-level absorption as a function of the excitation wavelength; and $a_{\text{II}}(\lambda_{\text{ex}})$ and $a_{\text{I}}(\lambda_{\text{ex}})$ are the relative absorption cross-sections for each population of photosystems as a function of the excitation wavelength ($0 \leq [a_{\text{II}}, a_{\text{I}}] \leq 1$). For example, a_{II} and a_{I} were found to remain rather constant in spinach leaves for the range 400–680 nm, but a_{I} rapidly increases to 1 at > 675–680 nm (Kitajima and Butler, 1975b; Butler, 1978) where PSII no longer absorbs. $F_{\text{PSII}}(\lambda_{\text{em}})$ and $F_{\text{PSI}}(\lambda_{\text{em}})$ are the

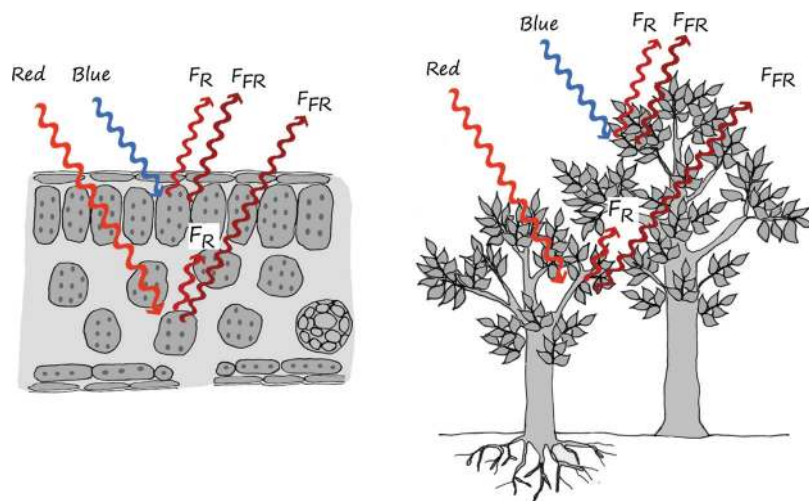


Fig. 7. Wavelength dependency of light penetration and fluorescence reabsorption within a leaf and a plant canopy. Red light penetrates deeper in the leaf or within a plant canopy compared with blue light due to higher scattering. In turn, red fluorescence (F_R) has a larger probability of being reabsorbed by chlorophyll within the leaf and canopy compared with far-red fluorescence (F_{FR}), due to the characteristics of the chlorophyll absorption spectra.

wavelength-dependent functions that account for the shape of the fluorescence emission spectra for PSII and PSI, respectively (Croce *et al.*, 1996; Franck *et al.*, 2002; Palombi *et al.*, 2011); and $p_r(\lambda_{em}, \text{Chl})$ is a function that accounts for fluorescence reabsorption and depends on fluorescence wavelength, chlorophyll content, and leaf morphology. In the following, we use Equation 12 to discuss the differences between PAM and SIF fluorescence.

4. Measuring chlorophyll fluorescence

Two main methods are used to measure ChlF in the field: those based on PAM systems (active methods) and those based on the retrieval of SIF (passive methods). Although active methods based on laser technology have been also developed (e.g. Kolber *et al.*, 2005; Pieruschka *et al.*, 2010), their applicability at the canopy and landscape level remains to be assessed. More generally, active methods are applied at the leaf level whereas passive methods are being commonly applied at the canopy level and beyond. In the following, we describe the background of these measurements, their differences, and some of the underlying challenges. For further details, we suggest reviews by Maxwell and Johnson (2000), Schreiber (2004), or Baker (2008) for PAM fluorometry, or that of Meroni *et al.*, (2009) for SIF.

4.1 Pulse amplitude-modulated fluorescence

The main feature of PAM fluorometers is that a weak and pulsed measuring light (ML) is used to excite fluorescence (Duysens, 1979). Widths of measuring pulses are in the microsecond range and the pulse amplitude is constant. Pulse frequencies can range from 10 Hz up to 200 kHz depending on fluorometer and instrument setting. At low frequencies, dark intervals between measuring pulses are relatively long so that the average photon flux density of the ML is very small ($<1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and does not cause any significant change in the degree of PSII openness (q) (see Section 3.2). PAM fluorometers subtract the fluorescence signal shortly before or after a measuring pulse from the fluorescence signal during this measuring pulse. This subtraction method is highly selective and eliminates virtually all non-PAM fluorescence from the measurement, for example the fluorescence excited by sunlight in field experiments. Because PAM fluorometers register only the fluorescence excited by this constant ML, variations in PAM fluorescence reflect variations in the efficiency by which the sample transforms modulated excitation light into fluorescence.

PAM fluorometers are characterized by the wavelength of the ML as well as the wavelength range at which modulated fluorescence is being registered. Recent advances in light-emitting diode (LED) technology allow PAM excitation in the entire visible range with a full width at half maximum (FWHM) between 20 nm and 40 nm. The spectral window for fluorescence detection is typically confined to wavelengths $>700 \text{ nm}$ using a long-pass colour filter, in order to minimize

reabsorption effects. Using such filters, the PAM ChlF signal is typically integrated over tenths of nanometres, providing a broadband measure of ChlF. These characteristics differ from instrument to instrument.

Using 'Equation 12, the PAM fluorescence signal (F') in relative units (e.g. mV) emanating from a leaf under illumination can be expressed as:

$$F' = \beta I_{\text{ML}}(\lambda_{\text{ML}}) A(\lambda_{\text{ML}}) \left[a_{\text{II}}(\lambda_{\text{ML}}) \frac{k_{\text{f}}}{k_{\text{f}} + k_{\text{D}} + k_{\text{NPQ}} + qLk_{\text{PSII}}} F_{\text{PSII}}(\lambda_{\text{retrieval}}) + a_{\text{I}}(\lambda_{\text{ML}}) \Phi F_{\text{I}} F_{\text{PSI}}(\lambda_{\text{retrieval}}) \right] [1 - p_r(\lambda_{\text{retrieval}}, \text{Chl})] \quad (13)$$

where β is a parameter that accounts for the properties and sensitivity of the detector system (e.g. $\text{mV m}^2 \text{s} \mu\text{mol photons}^{-1}$); $I_{\text{ML}}(\lambda_{\text{ML}})$ is the intensity of the PAM measuring light ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) with wavelength (λ_{ML}); $A(\lambda_{\text{ML}})$, $a_{\text{II}}(\lambda_{\text{ML}})$, and $a_{\text{I}}(\lambda_{\text{ML}})$ are the absorbance and relative absorption cross-section area of PSII and PSI, respectively, at the wavelength of the measuring light (λ_{ML}); and $F_{\text{PSII}}(\lambda_{\text{retrieval}})$ and $F_{\text{PSI}}(\lambda_{\text{retrieval}})$ account for the shape of the fluorescence emission spectra for PSII and PSI, respectively, averaged for the spectral range of the retrieval. ΦF_{I} is the fluorescence yield of PSI (see Equation 11) and $p_r(\lambda_{\text{retrieval}}, \text{Chl})$ the average reabsorption probability for the wavelength range at which fluorescence is being retrieved and for the prevailing leaf morphology and chlorophyll content.

Equation 13 can be greatly simplified as a result of PAM design and by using a number of assumptions. (i) Because PAM fluorometers typically (but not always) register fluorescence $>700 \text{ nm}$, we can assume they are not affected by reabsorption, i.e. $p_r(>700 \text{ nm}, \text{Chl})=0$. (ii) Since the amplitude of the ML is constant, we can assume $I_{\text{ML}}=\text{constant}$. Therefore, at constant A and a_{II} , F' is proportional to $\Phi F'$. (iii) The contribution of PSI fluorescence to the total signal is usually assumed to be constant or negligible. (iv) The characteristic emission spectra for PSII and PSI are assumed to remain constant for the duration of the measurements [$F_{\text{PSII}}(\lambda_{\text{retrieval}})$ and $F_{\text{PSI}}(\lambda_{\text{retrieval}})$]. Indeed, because PAM ChlF is acquired over a broadband, the resulting signal is less likely to be affected by seasonal shifts in the fluorescence emission spectra compared with SIF. (v) $A(\lambda_{\text{ML}})$ and $a_{\text{II}}(\lambda_{\text{ML}})$ are often assumed to remain constant or their variation negligible. Given these simplifications, F' in Equation 13 corresponds to ΦF_{II} in Equation 7.

The operating quantum yield of photochemistry in PSII (ΦP) can be obtained by comparing the fluorescence level in the presence (F') (Equation 7) and in the absence (F'_M) of photochemistry (Equation 8). PAM fluorometers estimate maximal fluorescence (F'_M) by providing a pulse of saturating light (SP) for several hundreds of milliseconds (typically 0.6–1 s). The SP momentarily reduces all the electron acceptors of PSII so that primary photochemistry tends to zero and $qL=0$, and thus $k_p=0$ (Bradbury and Baker, 1981; Schreiber *et al.*, 1986). As a result F' increases to a maximal level F'_M . Accordingly, ΦP can be then estimated from PAM data following Equation 9, as:

$$\Phi P = 1 - \frac{\frac{k_F}{k_D + k_F + k_{NPQ} + qLk_{PSII}}}{\frac{k_F}{k_D + k_F + k_{NPQ}}} = 1 - \frac{F'}{F_M} \quad (14)$$

Following from Equation 14 and provided that $I(\lambda)$, $A(\lambda)$, and $a_{II}(\lambda)$ are known for the range 400–700 nm (PAR), the rate of LET through PSII can be estimated as (Genty *et al.*, 1989; Schreiber, 2004; Baker, 2008):

$$ETR = I(\text{PAR})A(\text{PAR})a_{II}(\text{PAR})\Phi P \quad (15)$$

Commercial fluorimeters usually provide an estimate of the electron transport rate (ETR) by assuming that PAR leaf absorbance equals 0.84 (Baker, 2008) and that absorbed photons are equally distributed between the two photosystems (i.e. $a_{II}=0.5$). This approximation is reasonable for comparison of ETR values between optically similar samples such as leaves of cultivars of a single plant species. When the data expand over several species or extend to a long time period, then the significance of species-specific, within-canopy gradients or seasonal variation in A and a_{II} need to be taken into account when calculating ETR. The same applies to other fluorescence parameters (Baker and Oxborough, 2004; Logan *et al.*, 2007).

Other important fluorescence parameters can be estimated by dark-acclimating the leaf for a period of time (15 min to 2 h). The purpose of dark acclimation is to re-oxidize the Q_A electron acceptors in every PSII (which takes only several seconds) and to relax all the reversible NPQ, which may take a few minutes or hours, depending on temperature (Bilger and Björkman, 1991; Eskling, 1997; Demmig-Adams and Adams, 2006). By definition, dark acclimation of sufficient length decreases NPQ to zero, $k_{NPQ}=0$ in Equation 8. Under these conditions, minimal (F_0) and maximal (F_M) fluorescence levels of the dark-acclimated leaf are obtained.

Substituting F' by F_0 (i.e. qL approaches unity), and F_M' by F_M (i.e. NPQ approaches zero), we can use Equation 14 to estimate the maximum quantum yield of photochemistry (ΦP_{\max}), as (Kitajima and Butler, 1975a):

$$\Phi P_{\max} = 1 - \frac{F_0}{F_M} = \frac{F_M - F_0}{F_M} = \frac{F_V}{F_M} \quad (16)$$

The F_V/F_M parameter or ΦP_{\max} has been found to remain rather constant in non-stressed C_3 plants, with values of ~ 0.83 (Björkman and Demmig, 1987). Consequently, a decrease in F_V/F_M below the value found in healthy plants of the same species has been used as an indicator of decreased photochemical performance caused, for example, by photoinhibition of reaction centres or sustained forms of NPQ (Ottander, 1991; Adams and Demmig-Adams, 2004; Ensminger *et al.*, 2004; Porcar-Castell *et al.* 2008a, b).

Provided that factors such as light absorbance (A) and PSII relative absorption cross-section (a_{II}) remain constant, differences between F_M' and F_M can be used to estimate the regulated non-photochemical quenching or NPQ, via the parameter NPQ (Bilger and Björkman, 1991):

$$NPQ = \frac{k_{NPQ}}{k_F + k_D} = \frac{F_M - F_M'}{F_M'} \quad (17)$$

Similarly, F' , F_M' , and F_M can be combined to estimate the photochemical quenching or PQ, via the parameter PQ (Porcar-Castell, 2011):

$$PQ = qL \frac{k_{PSII}}{k_F + k_D} = \frac{F_M}{F'} - \frac{F_M}{F_M'} \quad (18)$$

These are just some of the many parameters that can be obtained using PAM fluorescence. Further photochemical and non-photochemical quenching parameters and process quantum yields can be found elsewhere (e.g. Roháček, 2002; Krause and Jahns, 2004; Schreiber, 2004; Baker, 2008; Porcar-Castell, 2011).

When the underlying assumptions are carefully considered (Maxwell and Johnson, 2000; Logan *et al.*, 2007; Baker, 2008), PAM fluorometry becomes a very versatile tool that provides highly informative data to track the acclimation of the light reactions of photosynthesis. PAM fluorometry also provides the possibility of studying in detail and modelling the link between ChlF and photosynthesis in the new spatiotemporal domain (seasonal, within-canopy, multiple species). We expect that the rapid spread of gas exchange systems combined with PAM fluorimeters, the recent availability of long-term PAM monitoring systems (Porcar-Castell *et al.*, 2008c; Porcar-Castell, 2011), and the development of other active techniques such as the laser-induced fluorescence transient (LIFT) (Kolber *et al.*, 2005; Pieruschka *et al.*, 2010) will favour these developments.

4.2 Solar-induced fluorescence and passive remote sensing

SIF is chlorophyll fluorescence that originates from excitations caused by the absorption of sunlight. SIF can be measured using passive remote sensing at a range of scales; from leaves in the laboratory (Gamon 1990) to satellite-based retrievals across the landscape (Guanter *et al.*, 2007; Frankenberg *et al.*, 2011; Joiner *et al.*, 2011). As discussed in the Section 4.1, PAM fluorimeters measure fluorescence induced by a (modulating) ML source which makes the observations independent from any other irradiance source (such as the sun). In contrast, careful characterization of the incident irradiance field is required if SIF-based ChlF observations are to be meaningfully compared with PAM fluorescence measurements.

The principal passive remote sensing technique used to estimate SIF is the Fraunhofer line depth/discriminator (FLD) method (Plascyk, 1975). Fraunhofer lines are dark, narrow regions of the solar spectrum that are caused by gaseous absorption in the Sun's photosphere. There are also several dark regions in the spectrum caused by gaseous absorption in the Earth's atmosphere, so-called telluric lines. Several Fraunhofer and telluric lines are found at wavelengths coincident with fluorescence emission and are literally filled in by fluorescence (examples are shown as grey vertical lines

on Fig. 8A). In reality, several spectrally adjacent lines may contribute to a specific Fraunhofer ‘feature’. It is across these features that the FLD algorithm is applied [in a slight abuse of terminology, we use ‘line(s)’ and ‘feature’ interchangeably in this section].

Changes in the fractional depths of Fraunhofer lines are used as the foundation of the FLD retrieval. In essence, the relatively weak fluorescence signal is amplified in these features of the spectrum. The FLD method can be applied to many different lines including the Fraunhofer hydrogen- α band (656 nm), potassium D1 (769 nm), the telluric oxygen-B (690 nm), and oxygen-A (760 nm) bands. To compute SIF using the FLD method it is not necessary to measure whole radiance or irradiance spectra; rather, four measurements are needed in total. One measurement of solar irradiance and target radiance is taken ‘inside’ the line, and one measurement of solar irradiance and spectral radiance is taken directly next

to the line. The following expression is used to retrieve fluorescence across the line (SIF _{$\lambda_{\text{retrieval}}$}):

$$SIF_{\lambda_{\text{retrieval}}} = \frac{L_{\text{inside}} E_{\text{outside}} - L_{\text{outside}} E_{\text{inside}}}{L_{\text{inside}} - E_{\text{outside}}} \quad (19)$$

L and E refer to radiance and irradiance measurements taken at a wavelength within (inside) and outside a particular line feature. The key assumption is that the bands are sufficiently close so that both inherent reflectance and fluorescence are constant across the lines. In practice, these assumptions are often violated, which has motivated the development of several improved FLD-based retrievals [see the description of the spectral fitting method below, and Meroni (2009) for an in-depth review of improved methods].

The physiological interpretation of the resulting SIF signal depends on the spectral line chosen for the retrieval, SIF _{$\lambda_{\text{retrieval}}$} . Consistent with PAM fluorescence (Equation 13), the SIF signal can be expressed using Equation 11 for a specific retrieval wavelength, as:

$$SIF_{\lambda_{\text{retrieval}}} = \int_{\lambda_{\text{sun}}=400}^{\lambda_{\text{sun}}=700} I(\lambda_{\text{sun}}) A(\lambda_{\text{sun}}) \left[a_{\text{II}}(\lambda_{\text{sun}}) \Phi F_{\text{PSII}}(\lambda_{\text{retrieval}}) + a_{\text{I}}(\lambda_{\text{sun}}) \Phi F_{\text{PSI}}(\lambda_{\text{retrieval}}) \right] \left[1 - p_r(\lambda_{\text{retrieval}}, \text{Chl}) \right] d\lambda_{\text{sun}} \quad (20)$$

From Equation 20 it is evident that the interpretation of the resulting SIF signal depends on the spectral window chosen to retrieve SIF. The SIF signal measured at the O₂B line, SIF₆₉₀, is very close to the chlorophyll absorption peak and is therefore very sensitive to reabsorption of fluorescence by chlorophyll. This is not the case for SIF₇₆₀ which is measured in the O₂A line. Therefore, the SIF signal carries different physical–physiological information depending on the region used in the retrieval (Fig. 7). In addition, the contribution from PSI fluorescence will also vary depending on the retrieval line wavelength. This is due to differences in wavelength dependencies of $F_{\text{PSII}}(\lambda_{\text{retrieval}})$ and $F_{\text{PSI}}(\lambda_{\text{retrieval}})$ in Equation 20 which cause a higher relative contribution of PSI fluorescence at longer wavelengths since $F_{\text{PSII}}(690) \gg F_{\text{PSII}}(760)$ (Fig. 6). A further challenge faced when retrieving SIF from space is accurately correcting for atmospheric scattering and absorption effects. These may act to modify the observed SIF signal. In particular, when using telluric lines to retrieve SIF, the same chemical species that absorbs solar irradiance in the Earth’s atmosphere (causing the feature) also absorbs the emitted SIF signal. This means that precise atmospheric correction is essential if accurate canopy-leaving estimates of SIF are to be retrieved from at-sensor observations.

A promising development of the FLD method is the spectral fitting method (SFM) (Meroni *et al.*, 2009). The SFM solves the inverse problem of estimating fluorescence across a Fraunhofer feature by using a least-squares approach. In the SFM, FLD observations are fitted with synthetic spectra modelled using simple polynomial functions. The SFM was designed to reduce noise and to be more robust to contamination by atmospheric effects than the FLD method and was demonstrated to increase the accuracy of SIF retrievals in comparison with the basic FLD algorithm (Meroni *et al.*, 2009).

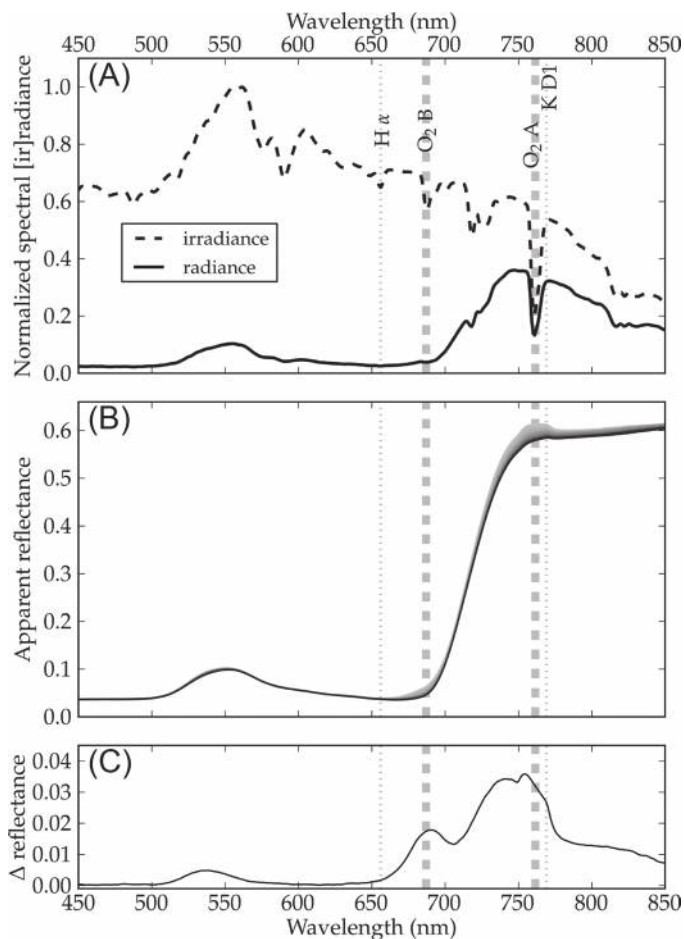


Fig. 8. Leaf spectral reflectance including the solar-induced fluorescence contribution. Incoming solar irradiance and spectral radiance of a maple (*Acer* sp.) leaf, measured using an ASD-HH Field Spec spectroradiometer (ASD Inc., USA). Hydrogen and oxygen absorption bands are highlighted in grey (A). Temporal dynamics in spectral reflectance measurements upon exposure of a dark-adapted maple leaf to full sunlight over 3 min. The rapid fluorescence dynamics are shown in grey, and the black line denotes the steady-state spectrum after a few minutes of illumination (B). (C) The difference between the first and last reflectance spectra from the middle panel. The large peak in the difference spectrum in the near-infrared wavelengths denotes the spectral properties of the dynamic (variable) fluorescence (J. Atherton *et al.*, unpublished data).

In addition to FLD-based methods, reflectance spectroscopy can be used to gain insight into the relationship between ChlF and photosynthesis. This is because the fluorescence emission spectrum is superimposed on the leaf (or canopy) reflectance spectrum. The reflectance spectrum is measured, typically in the laboratory or the field, as the ratio of reflected radiance to incident irradiance using hyperspectral spectroradiometers (Fig. 8B). A number of reflectance band ratios (algebraic combinations of ≥ 2 narrow spectral reflectance ‘bands’) have been developed to quantify the effect of fluorescence emission on the reflectance spectrum (Meroni *et al.*, 2009). These methods are particularly popular at the leaf scale where they are often compared and contrasted to reflectance indices that measure related physiological processes such as the photochemical reflectance index (PRI) (Gamon *et al.*, 1992) or chlorophyll-based indices (Richardson and Berlyn, 2002). Reflectance difference spectra are useful as measures of the dynamic fluorescence contribution to reflectance. Difference spectra can be calculated from sets of reflectance spectra both as a function of time (Gamon *et al.*, 1990) and using a filter-based approach (Zarco-Tejada *et al.*, 2000; Campbell *et al.*, 2008; Meroni *et al.*, 2009). Filter-based approaches are used to extricate the chlorophyll fluorescence contribution from the measured apparent reflectance spectrum.

5. Remaining challenges: from diurnal to seasonal, from the leaf to the landscape, from active to passive fluorescence.

Time-resolved fluorescence spectra and lifetime analysis have proved essential for elucidating structural and functional features of photosynthesis (Roelofs *et al.*, 1992; Dau, 1994; Govindjee, 1995; Croce *et al.*, 1996; Lázár, 1999; Jennings *et al.*, 2003; Vassiliev and Bruce, 2008). Likewise, PAM fluorescence measurements have been valuable in studying the ecophysiology of photosynthesis at the leaf level and *in situ* (Bradbury and Baker, 1991; Ottander *et al.*, 1991; Adams and Demmig-Adams, 1994; Verhoeven *et al.*, 1996; Ogaya and Peñuelas, 2003; Ensminger *et al.*, 2004; Porcar-Castell *et al.*, 2008a, b, c; Porcar-Castell, 2011).

The ChlF signal is now being applied to the study of photosynthesis from remote sensing platforms. Before we can mechanistically link SIF and GPP, we need to expand the knowledge we have gained from short-term measurements using mainly PAM techniques at leaf level, to the seasonal and canopy level using SIF instead. This involves a number of challenges that are introduced in this section and can be summarized as follows. (i) Temporal up-scaling. Factors that control the seasonal variation of ChlF properties remain unclear. (ii) Spatial up-scaling. In addition to characterizing and modelling the radiative transfer of the signal, up-scaling from the leaf to the landscape also requires understanding of the vertical and species-specific variation in physiological traits, an area with very limited information. (iii) Mechanistic up-scaling from SIF to gross photosynthesis. In contrast to PAM fluorescence, the quantum yield of

photochemistry cannot be directly resolved from SIF. This problem can only be bypassed by developing robust models to link SIF to GPP, an area where a quantum leap forward is urgently needed.

5.1 Temporal up-scaling: from the diurnal to the seasonal scale

Over the short term (seconds to days) it is reasonable to assume that most of the factors that control the ChlF signal at the leaf level (Equation 12) remain constant, so that variations in fluorescence can be largely attributed to photochemical or non-photochemical processes in PSII (i.e. q_L and NPQ) (Maxwell and Johnson, 2000; Logan *et al.*, 2007; Baker, 2008), as well as to changes in illumination $I(\lambda_{ex})$ in the case of SIF. However, it remains largely unknown whether these simplifying assumptions hold at the seasonal scale (days to months).

Changes in chlorophyll contents, leaf absorptance, and fluorescence reabsorption

Leaf chlorophyll concentrations vary at the seasonal time scale (García-Plazaola and Becerril, 2001; Lu *et al.*, 2001). This is particularly evident during leaf development and senescence, but adjustments in pigment concentrations occur also in evergreen foliage during the course of the seasons. For example, boreal Scots pine trees decrease their needle chlorophyll concentration by up to ~40% in winter compared with summer (Ensminger *et al.*, 2004; Porcar-Castell *et al.*, 2012). An increase in leaf-level chlorophyll content may have mixed effects on ChlF.

Chlorophyll concentration modulates light absorptance $A(\lambda)$ and, by extension, ChlF (Equation 12). The relationship between chlorophyll content and light absorptance is positive and non-linear, saturating at high chlorophyll contents (Björkman and Demmig, 1987; Adams *et al.*, 1990; Gitelson *et al.*, 1998). In agreement with this, the relationship between leaf-level chlorophyll concentration and the fluorescence signal also saturates at high chlorophyll contents (Adams *et al.*, 1990).

Similarly, chlorophyll concentration also modulates fluorescence reabsorption (Equation 12) and, by extension, ChlF. An increase in absorption $A(\lambda)$ will cause an increase in fluorescence at all wavelengths, whereas an increase in reabsorption will decrease red but not far-red ChlF (Lichtenthaler and Rinderle, 1988; Gitelson *et al.*, 1998). This phenomenon is built in the ratio of red to far-red fluorescence (F_R/F_{FR}) to estimate chlorophyll concentrations. Interestingly, the processes controlling F_R/F_{FR} depend on scale. At the diurnal scale, F_R/F_{FR} varies in response to the action of NPQ (Agati *et al.*, 1995), whereas at the seasonal scale or when comparing leaves with different chlorophyll contents, F_R/F_{FR} reflects changes in chlorophyll concentration (Lichtenthaler and Rinderle, 1988; Gitelson *et al.*, 1998). This scale-dependent relationship also affects the correlation between F_R/F_{FR} and LUE. The ratio F_R/F_{FR} is directly proportional to LUE over the diurnal scale (Agati *et al.*, 1995), but becomes inversely proportional to LUE at the seasonal scale (Freedman

et al., 2002), emphasizing the importance of scale when interpreting data.

Leaf chlorophyll concentration is therefore expected to have a mixed effect on the ChlF signal, with absorption effects dominating in leaves with low chlorophyll concentrations (i.e. more chlorophyll more fluorescence), and reabsorption effects dominating in leaves with high chlorophyll contents (i.e. more chlorophyll less fluorescence) (unless PAM fluorometer design discriminates all red fluorescence). This is consistent with results obtained in *Hedera canariensis* and *Platanus occidentalis* where the shift from absorption- to reabsorption-dominated effects took place at chlorophyll concentrations around 250 mg Chl m⁻² (Björkman and Demmig, 1987; Adams *et al.*, 1990). Further characterization of the interplay between chlorophyll contents, leaf absorption, and wavelength-dependent fluorescence properties across leaves with different morphologies is warranted to model these effects

Changes in relative absorption cross-sections of PSII (a_{II}) and PSI (a_I)

PSII and PSI have different fluorescent properties. The quantum yield of PSI (ΦF_I) is typically much smaller than ΦF_{II} , and PSI fluorescence peaks at longer wavelengths than PSII fluorescence (Fig. 6). Consequently, changes in the relative absorption cross-sections of PSII (a_{II}) and PSI (a_I) affect the fluorescence yield and spectra of the leaf. Over the short term, state transitions are known to reduce a_{II} and increase a_I , and, since $\Phi F_I < \Phi F_{II}$, the intensity of the fluorescence signal decreases. This quenching has been described in PAM fluorescence terms as qT (Krause and Weis, 1991), although qT does not contribute to NPQ as such (non-radiative quenching of excitation energy in PSII) but simply quenches F' by decreasing a_{II} , which results in a decrease in F_M' (Equation 8) and a subsequent increase in the parameter NPQ (Equation 17). In addition, state transitions also produce a change in the spectral properties of the F signal, with F_R being decreased to a larger proportion than F_{FR} due to a larger contribution of PSII fluorescence in the red relative to far-red. This phenomenon is particularly visible at liquid nitrogen temperature (77 °K) when the PSII and PSI fluorescence peaks can be separated, with F_R (originating mainly in PSII) and F_{FR} (originating mainly in PSI) (Butler, 1978; Govindjee, 1995). Changes in the 77 °K fluorescence ratio F_R/F_{FR} have been used to study state transitions (e.g. Tan *et al.*, 1998).

The question is, how stable/dynamic are the relative absorption cross-sections at the seasonal scale? If a_{II} and a_I would change over the season, we could expect a similar effect to that of state transitions, and possibly modifications in the relationship between ChlF, NPQ, and photochemistry. Direct measurement of the relative absorption cross-sections of PSII and PSI remains a challenge (Laisk *et al.*, 2002; Eichelmann *et al.*, 2005). Studies that directly measure a_{II} and a_I in conjunction with fluorescence spectral properties at the seasonal scale will help elucidate the dynamics and impact of a_{II} and a_I on ChlF.

Down-regulation of photosynthesis

Leaf-level fluorescence spectra might also change in response to the structural re-organization of the thylakoid observed, for example, in response to low temperature. The aggregation of LHCs in PSII has been suggested to be part of the mechanism of sustained stress-induced down-regulation of PSII (Ottander *et al.*, 1995; Ensminger *et al.*, 2004; Verhoeven, 2014). Winter down-regulation of the photosystem is known to be accompanied by up-regulation in SpsB proteins and xanthophyll cycle pigments (Ensminger *et al.*, 2004; Zarter *et al.*, 2006). These sustained quenching phenomena enhance NPQ and decrease the quantum yield of fluorescence and photochemistry, but also induce changes in fluorescence spectra. Gilmore and Ball (2000) identified what they called the cold-hard band (CHB) in overwintering snow gum leaves, where they showed that the leaf fluorescence spectrum was blue-shifted (i.e. moved towards shorter wavelengths) in response to cold acclimation. Interestingly, the CHB was shown to be fully reversible under constant chlorophyll content during recovery, suggesting that NPQ and perhaps also fluorescence spectra of the photosystems, $F_{PSII}(\lambda_{em})$ and $F_{PSI}(\lambda_{em})$, modulate the fluorescence spectral properties during down-regulation episodes. Measuring the fluorescence spectral properties of leaves and isolated PSII units under different levels and forms of NPQ (i.e. reversible and sustained) could serve to study how fluorescence spectra change in response to down-regulation of photosynthesis and its impact on both PAM and SIF fluorescence.

Effect of temperature

Long-term field observations (such as those in remote sensing) are often conducted under a wide range of temperatures that can easily exceed 60 °C. The yield and shape of the fluorescence spectrum are known to change in a thermodynamic process that takes place independently of plant physiological status (Croce *et al.*, 1996; Gobets and van Grondelle, 2001). When temperature is decreased from ambient to liquid nitrogen temperature (77 °K) the fluorescence yield of PSI ΦF_I increases because excitations are trapped in the so-called red chlorophylls (Croce *et al.*, 1996; Gobets and van Grondelle, 2001; Jennings *et al.*, 2003).

Red chlorophylls absorb at longer wavelengths than the rest of the antenna, but, at physiological temperatures, uphill energy transfer from the red chlorophylls to the bulk antenna is possible through thermal activation using vibrational energy from the phonon bath (i.e. energy contained in the vibrations of the pigment-protein matrix) (Jennings *et al.*, 2003; van Grondelle and Novoderezhkin, 2006). At low temperature (e.g. 77 °K), the phonon bath does not provide sufficient energy for the uphill energy transfer (Jennings *et al.*, 2003), and excitations get trapped in these chlorophylls, giving rise to an increase in fluorescence yield as well as a slight red shift in peak position (Croce *et al.*, 1996). Low temperature also limits energy transfer between PSII proteins, and two spectral peaks or shoulders (685 nm and 695 nm) appear at 77 °K instead of one single peak observed at room temperature (Govindjee, 1995; Keränen *et al.*, 1999; Maxwell and Johnson, 2000).

Because these thermodynamic effects take place gradually between ambient and liquid nitrogen temperature (Brody and Brody, 1962; Gylle *et al.*, 2012), they may need consideration when analysing ChlF data sets collected under a wide range of temperatures. For example, the ChlF of a *Chlorella* sp. suspension increased by 30% when lowering temperature from 25 °C to -30 °C at 720 nm (Brody and Brody, 1962). In addition, the differential dependence of both F_0 and F_M on temperature at a physiologically relevant range has also been observed in leaves (Weis and Berry, 1988; Kuropatwa *et al.*, 1992; Pospíšil *et al.*, 1998). Similar increases in ChlF in both the red and near infrared with decreasing temperature have been observed in intact leaves (e.g. Agati, 1998; Agati *et al.*, 2000). Physiological and thermodynamic effects cannot always be decoupled empirically, although physiological factors determine the differential effect on F_0 and F_M (Pospíšil *et al.*, 1998) or the dependence of the temperature effect on the needle age found in spruce (Ilík *et al.*, 1997). The impact and seasonal dynamics of the thermodynamic phenomena deserve to be quantified and characterized with dedicated experiments using various species and leaves presenting different physiological states, so that the effect can be modelled and corrected if needed.

Seasonal changes in connectivity between PSII units

Connectivity is the phenomenon by which excitation energy moves between neighbouring photosystems. This affects the relationship between fluorescence and the fraction of open/functional reaction centres (q) (Fig. 5) (Joliot and Joliot, 1964; Havaux *et al.*, 1991; Lavergne and Trissl, 1995; Kramer *et al.*, 2004b). In the absence of connectivity (separate units model), fluorescence yield is linearly related to q (Fig. 5A). In contrast, a system with connected PSII units will result in lower fluorescence yield for the same levels of reaction centre closure (q), because excitation energy in closed reaction centres can be quenched by neighbouring open reaction centres. Therefore, changes in connectivity *per se* can generate a change in ΦF at constant q and NPQ, complicating the interpretation of ChlF data. Connectivity between PSII units has been shown to change in response to protein phosphorylation (Kyle *et al.*, 1982), salt stress (Mehta *et al.*, 2010), and thylakoid grana stacking (Chow *et al.*, 2005). Because the impact of connectivity on ChlF decreases with NPQ (Fig. 5A–D), and because SIF is by default measured under illumination in the presence of NPQ, it is likely that connectivity and its potential dynamics do not exert a significant control on SIF. However, because seasonal changes in connectivity are likely to occur, the lake model assumption requires validation at the seasonal scale. The potential impact of seasonal changes in connectivity needs to be considered when attributing changes in photochemical quenching parameters such as qP or qL to processes such as photoinhibition of reaction centres, or when a connectivity assumption is used to derive fluorescence parameters as in the LIFT approach (Kolber *et al.*, 2005).

The contribution of PSI fluorescence to total fluorescence
The dynamics of the ChlF signal are most often associated with PSII and used to derive information on the acclimation

of PSII. PSI has been typically ignored or corrected for using a constant offset value. Using different techniques and species, the contribution of PSI fluorescence has been found to range from close to zero in the red portion of the fluorescence spectra and up to 50% (Genty *et al.*, 1990b; Dau, 1994; Pfündel, 1998; Agati *et al.*, 2000; Peterson *et al.*, 2001; Franck *et al.*, 2002; Palombi *et al.*, 2011; Pfündel *et al.*, 2013). Franck *et al.* (2002) presented a method to resolve the spectral fluorescence contribution at the F_0 and F_M states (Fig. 6) that illustrates the wavelength dependency of the PSI fluorescence contribution. The contribution of PSI to total fluorescence decreases with decreasing photochemical quenching in PSII, becoming relatively insignificant at the F_M level ($q=0$). Under natural illumination F' and SIF levels are typically much closer to F_0 than to F_M , and the contribution that should be taken as reference of PSI fluorescence 'offset' is that obtained at the F_0 level and not the F_M . Clearly, PSI contribution to total fluorescence is important and cannot be ignored. However, can we expect this ChlF 'offset' to remain constant over time?

There are three different mechanisms by which PSI fluorescence contribution can vary. (i) Changes in chlorophyll content affect reabsorption of F_R (Lichtenthaler and Rinderle, 1988; Gitelson *et al.*, 1998). Consequently, because the red region is enriched in PSII fluorescence (Fig. 6), increasing chlorophyll contents will tend to increase the overall PSI fluorescence contribution. (ii) PSI contribution might dramatically increase in response to sustained NPQ and down-regulation of PSII. Although F_0 represents a theoretical minimal fluorescence, fluorescence is known to decrease below summer/non-stressed F_0 levels when the photosystems are deeply down-regulated (Porcar-Castell *et al.*, 2008a; Soukupová *et al.*, 2008; Porcar-Castell, 2011). Under these conditions, and unless sustained forms of NPQ operate in a similar fashion in both photosystems, the contribution of PSI fluorescence to the total signal could dramatically increase relative to the contribution estimated at the summer F_0 . Finally, (iii) seasonal changes in the relative absorption cross-sections of PSII and PSI would equally affect PSI contribution. The seasonal dynamics in the PSI contribution to the total ChlF signal await further study so that we can relate the seasonal changes in the ChlF signal to processes taking place only in PSII, or in both photosystems.

In particular, the significance of sustained forms of NPQ in PSI and its putative impact on ΦF_1 remain controversial. On one hand, there is no evidence of a significant effect of NPQ on PSI fluorescence over the short term (Genty *et al.*, 1990b; Krause and Jahns, 2004; Pfündel *et al.*, 2013), although it is known that 30–50% of the xanthophyll cycle pigments are associated with PSI, and de-epoxidation reactions also take place in PSI (Thayer and Björkman, 1992; Lee and Thornber, 1995; Färber, 1997). In contrast, NPQ has been found to have no effect on the redox state of the electron transport chain (Tikkanen *et al.*, 2011), which suggests that NPQ might operate in an equal fashion in both photosystems, unless CET is highly dynamic. The hypothesis of a constant ΦF_1 awaits validation at the seasonal scale.

5.2 Spatial up-scaling

With the development of ChlF measurement techniques that can monitor targets of different size (leaf, canopy, and landscape), the question of scaling of process knowledge becomes increasingly important (Fig. 4). One of the main challenges in up-scaling is how to represent the spatial variability in physical and physiological factors within a leaf or a plant canopy (Malenovsky *et al.*, 2009). When measuring SIF emitted by a leaf, the resulting signal can be expected to emanate predominantly from the top layers which are exposed to higher irradiance levels, as discussed in Section 3.3. Similarly, when measuring SIF of a plant canopy, the SIF signal will mainly consist of fluorescence originating from the top of the canopy, which is exposed to higher irradiance. For canopy-level observations, an additional problem is that the signal depends on canopy structure, sun elevation, and view angle, with different fractions of sunlit and shaded leaves visible from the perspective of the observer when looking at the same canopy from different angles. SIF thus depends on the position of the observer, whereas the emission at the photosystem level does not (van der Tol *et al.*, 2009).

Similarly, as discussed in Section 3.3, the fact that the resulting fluorescence signal needs to travel through a variable depth of leaf or canopy before it reaches the sensor will change the spectral properties of the signal due to variable and selective reabsorption of red fluorescence (Gitelson *et al.*, 1998; Franck *et al.*, 2002). The signal will be enriched in top canopy leaves for SIF (red), for example SIF₆₉₀, with a larger contribution from leaves deeper in the canopy for SIF (far-red), for example SIF₇₆₀. Daumard *et al.* (2012) analysed measurements of top-of-canopy SIF in a crop in the red and far-red during an entire season, and concluded that the far-red contains clear information about the development and photosynthetic capacity of the crop, whereas the F_R/F_{FR} ratio was sensitive to chlorophyll concentrations and the structure of the vegetation.

Radiative transfer models have been developed to simulate the effects of leaf and canopy structure and geometry on the wavelength-dependent radiative fluxes, ranging from simple turbid medium approaches (Allen, 1964) to ray tracing in realistic vegetation models (Gastellu-Etchegorry *et al.*, 2004). Of intermediate complexity (and realism) are models that treat the canopy as a stochastically distributed arrangement of leaves (Verhoef, 1984). Some of these models also include the radiative transfer of ChlF emission in leaves (Pedrós *et al.*, 2010) and canopies (Rosema *et al.*, 1998; Miller *et al.*, 2005; van der Tol *et al.*, 2009; Zarco-Tejada *et al.*, 2013). These models are designed to reproduce the fate of the emitted ChlF spectra through the leaf and canopy. It is not yet known how accurately these models represent the structural effects of vegetation on observations of SIF, or how sensitive they are to the underlying assumptions of leaf and canopy structure.

In addition to understanding radiative transfer as a physical process (physical modelling), we also need to solve the question of how physiological traits and photosynthesis dynamics (Equation 12) control the resulting fluorescence signal (physiological modelling). Understanding this aspect requires coupling radiative transfer with physiology. Models that couple radiative transfer and photosynthesis have been used for

decades (Sellers, 1985; Goudriaan, 1988), but SIF has been only recently included (Miller *et al.*, 2005; van der Tol *et al.*, 2009). Even though these models have already been implemented for certain real-world scenarios, it remains unknown how well they represent the interactions between illumination and physiology (Malenovsky *et al.*, 2009), and whether they are meaningful to spatiotemporal scales of remote sensing (i.e. seasonal scale, and canopy/landscape level with multiple species). In particular, the temporal variation of leaf-level traits discussed above (Section 5.1), the vertical variation in leaf-level traits such as leaf absorbance (Ellsworth and Reich, 1993), PSII:PSI stoichiometry, and relative absorption cross-sections (Chow *et al.*, 1990; Rivadossi *et al.*, 1999) observed within a plant canopy, and the variation of leaf-level traits across species, need to be characterized and implemented within these models.

5.3 Mechanistic up-scaling: from SIF to GPP

Despite the complexity of the relationship between SIF and GPP, and despite the limited spatial and temporal resolution of the currently available remotely sensed SIF data (10 km, monthly) the resemblance of global maps of satellite-retrieved SIF and modelled GPP (Frankenberg *et al.*, 2011; Joiner *et al.*, 2011; Guanter *et al.*, 2012) shows that the relationship between satellite-derived SIF and GPP is significant, albeit probably biome dependent. The question remains of whether we have enough process understanding to explain the correlation between SIF and GPP mechanistically. How can we assimilate SIF into models to improve/benchmark spatially distributed GPP estimates? The current practice of estimating GPP from remote sensing is to parameterize LUE (Equation 1) by means of either statistical (Xiao *et al.*, 2004; Jung *et al.*, 2011) or physically based models (e.g. Ryu *et al.*, 2011). In both approaches, a combination of flux tower data and remote sensing products are used.

An important question when considering Equation 1 is whether SIF can be used to infer LUE, APAR (where $APAR = PAR \times fAPAR$), or both. Remote sensing of green $fAPAR$ based on reflectance measurements remains a challenge due to signal saturation, bidirectional reflectance distribution function (BRDF) effects (Morton *et al.*, 2014), and because materials such as soil, wood, and dead biomass also absorb PAR but do not contribute to photosynthesis (Huete, 1988; Qi *et al.*, 1994; Daughtry *et al.*, 2000; Haboudane *et al.*, 2002). In contrast, because ChlF originates only from chlorophyll, SIF could improve the estimation of $fAPAR$ in comparison with current reflectance-based methods (Frankenberg *et al.*, 2011).

The question of whether SIF is a measure for LUE is difficult to answer. In contrast to PAM measurements, it is not obvious how SIF (Equation 20) can be related to photochemistry. The information in a measurement of SIF is simply insufficient to calculate the quantum yield of photochemistry as derived from Equation 9 (unknown F_m' when measuring SIF). The accumulated knowledge provided by PAM fluorescence measurements may provide a path to understanding and modelling the relationship between SIF, electron transport, and GPP.

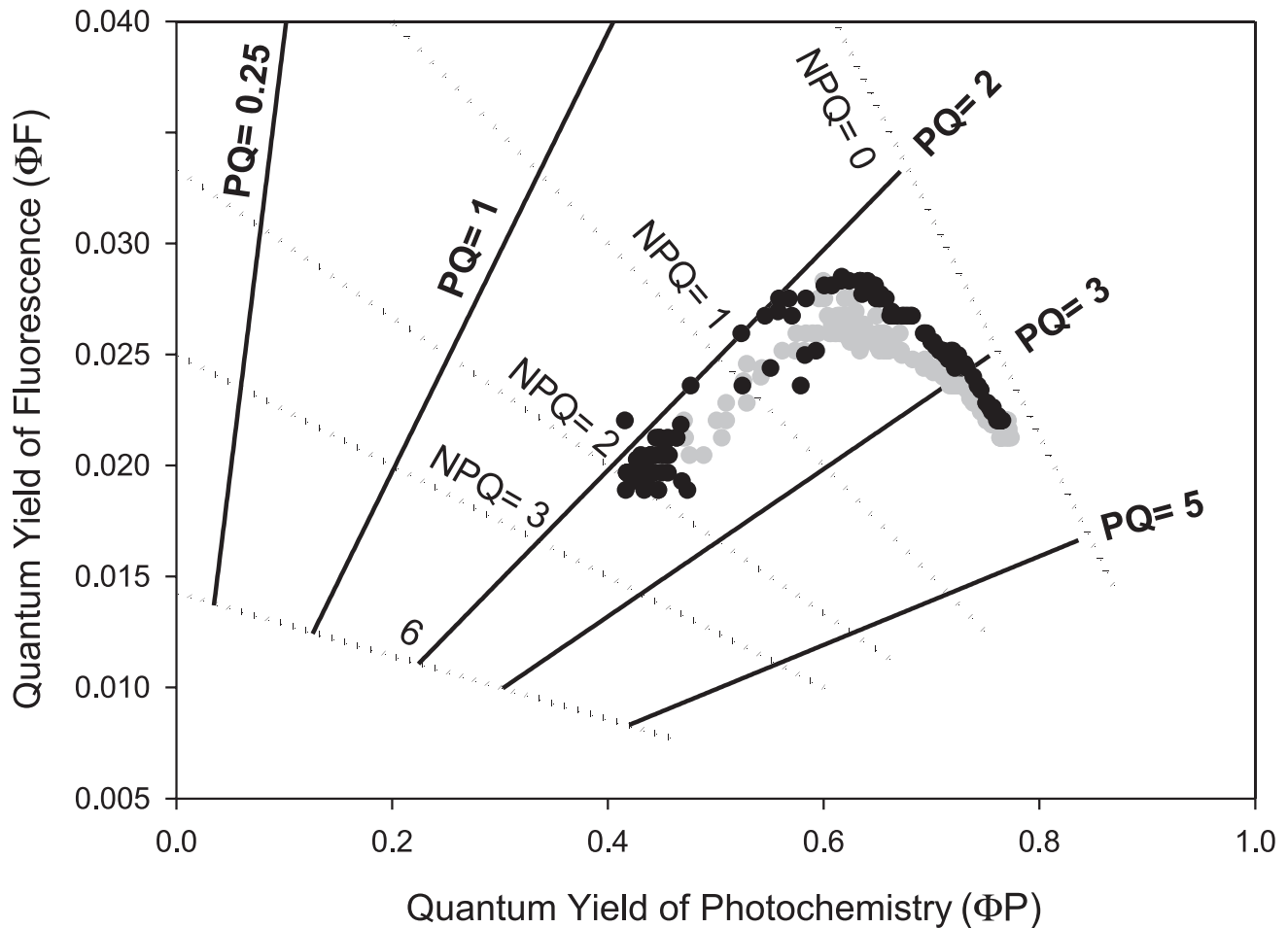


Fig. 9. Relationships between the quantum yields of photochemistry (Φ_P) and fluorescence (Φ_F) in relation to photochemical (PQ) and non-photochemical quenching (NPQ) of excitation energy. Lines were obtained by assuming a maximum quantum yield of fluorescence at the F_M state of 10% [i.e. $k_F=0.1(k_F+k_D)$], and by varying PQ and NPQ to estimate $\Phi_F=0.1/(1+PQ+NPQ)$ and $\Phi_P=PQ/(1+PQ+NPQ)$. It can be appreciated that under constant NPQ (dotted lines), fluorescence and photochemical yield are inversely proportional, whereas at constant PQ (solid lines), fluorescence and photochemical yield become proportional. Data were obtained during a summer day and using a Monitoring PAM fluorometer (Walz GmbH, Germany); data are from Porcar-Castell *et al.* (2008c). Black points correspond to data from midnight to noon, grey points from noon to midnight.

Fluorescence (Φ_F) and photochemical (Φ_P) yields (Equations 7 and 9, respectively) are affected by photochemical (PQ) and non-photochemical quenching (NPQ) (see Equation 10 and Fig. 9). In the field, the quantum yield of fluorescence is highly dynamic, both during the course of a day (Porcar-Castell *et al.*, 2008b) and throughout seasons (Soukupová *et al.*, 2008; Porcar-Castell, 2011), where Φ_F decreases during stress episodes and increases upon recovery in a process that appears to be largely controlled by the presence of sustained NPQ forms (Ensminger *et al.*, 2004; Porcar-Castell *et al.*, 2008a; Porcar-Castell, 2011). Therefore, Φ_F is related to Φ_P and hence to LUE. In conclusion, SIF contains information relating to LUE as well as APAR. Yet, disentangling these two contributions remains a challenge from a remotely sensed large-scale observation platform.

Over the course of a day, the relationship between Φ_F and Φ_P falls apart into two distinct phases: under low light (first morning hours and towards sunset) the changes in the quantum yield of photochemistry are controlled by PQ, with

NPQ remaining approximately constant and low (Fig. 9). In contrast, under high light (noon hours) the changes in Φ_P are dominated by NPQ, with PQ remaining rather constant. Since decreasing PQ and increasing NPQ have opposite effects on Φ_F (see Equation 7), this non-complementary behaviour generates a two-phased inverted 'V' relationship between Φ_P and Φ_F (Fig. 9), where Φ_P and Φ_F are inversely proportional under low light 'PQ-Phase', and proportional under high light 'NPQ-Phase'. The inverted 'V' relationship can be reproduced using current process-based understanding of the relationship between ChlF and photosynthesis (see Fig. 2 in van der Tol *et al.*, 2009).

Remotely sensed SIF will always be obtained under high-light conditions ('NPQ-Phase'); therefore, it could be expected that SIF and Φ_P vary concomitantly in response to stress, with $SIF \sim \Phi_P$ APAR. Indeed, a good correlation between Φ_F and photosynthesis has been observed in response to water stress episodes (Flexas *et al.*, 2000) when measured under high light in the 'NPQ-Phase'. Similarly, both Φ_F and Φ_P tend to decrease simultaneously in response

to low temperatures due to the action of sustained NPQ (Soukupová *et al.*, 2008; Porcar-Castell, 2011). As a caveat, it is likely that the sensitivity of the relationship changes during the season in response to sustained NPQ or photoinhibition of reaction centres, something that remains to be clarified and awaits validation. For example, the hysteresis observed in Fig. 9 between morning and afternoon observations may indicate that these processes already play a role at the diurnal scale. Overall, the general relationship between ΦP and ΦF presented in Fig. 9 demonstrates the strong control of the fluorescence signal by the dynamics of PQ and NPQ. This link has been described with empirical relationships that have been parameterized in radiative transfer models (Lee *et al.*, 2013).

To understand further how ΦF and ΦP , as well as ΦF and SIF, are related at the seasonal scale, the following points need consideration. (i) Over the short term, NPQ is known to decrease both SIF and F' , although for SIF the effect is known to be stronger when estimated in the red region (e.g. SIF₆₉₀) compared with the far-red (e.g. SIF₇₆₀) (Lichtenthaler and Rinderle, 1988; Agati *et al.*, 1995), most probably due to the NPQ-insensitive contribution of PSI far-red fluorescence. At the seasonal scale, the PAM term F' may decrease in response to stress due to accumulation of sustained forms of NPQ, yet, because stress is often accompanied by a decrease in chlorophyll content (i.e. decreased fluorescence reabsorption), SIF₆₉₀ (retrieved in the red region) may actually increase instead of decreasing like F' . (ii) Similarly, seasonal changes in the contribution of PSI may equally interact with the expected relationship between ΦF and ΦP when using SIF₇₆₀ (retrieved in the far-red) instead. Further long-term experimental work is needed to characterize the processes that control the relationship between SIF_{λretrieval}, F' , ΦF , and ΦP at the seasonal scale. Last, but not least, the seasonal dynamics of alternative electron sinks, alternative metabolic pathways, and photorespiration (Fig. 1) will need characterization before we can mechanistically link SIF and GPP.

6. Concluding remarks

The availability, quality, and spatiotemporal coverage of SIF data are expected to increase drastically over the next few years. At the canopy scale, field spectroradiometer prototype systems are being rapidly developed and deployed in increasing numbers (Rossini *et al.*, 2010; Balzarolo *et al.*, 2011; Drolet *et al.*, 2014). These systems will promote the expansion of SIF measurements at the ecosystem scale. The increased knowledge will hopefully lead to a better understanding of the SIF signal and its relationship to GPP. In addition, airborne instruments specifically designed to map vegetation fluorescence have recently been developed (Rascher *et al.*, 2009; Zarco-Tejada *et al.*, 2009, 2012). This opens the way for extensive airborne campaigns to better understand the significance of spatial variability in the SIF signal and facilitate the up-scaling from the canopy to the landscape levels.

Finally, and most significantly, imminent space missions will herald a new range of possibilities for retrieving SIF at the global scale. This could result in improved estimates of the global carbon budget and our capacity to track the health of terrestrial ecosystems. In particular, the ESA's Earth Explorer Fluorescence Explorer (FLEX) (Moreno *et al.*, 2006; Kraft *et al.*, 2013) is expected to provide an unprecedented source of spatially continuous SIF data as well as other critical variables to facilitate the signal interpretation (e.g. photochemical reflectance index, canopy temperature, f APAR). Likewise, satellite missions such as the NASA OCO-2 (Frankenberg *et al.*, 2014) or the ESA Sentinels 4–5, sensors conceived to measure atmospheric properties, will also offer exciting possibilities to retrieve SIF. Despite this promising scenario and despite the empirical evidence that confirms that SIF carries novel information on the dynamics of photosynthesis compared with previous remote sensing data (Frankenberg *et al.*, 2011; Joiner *et al.*, 2011; Guanter *et al.*, 2012), the mechanistic link between SIF and GPP still remains unclear.

In this review, we identified a number of areas where further research is deemed necessary to understand fully the mechanisms that control the seasonality of the SIF signal at the leaf level. Three prospective research areas for future work can be summarized.

- (i) Characterization of processes that uncouple ChlF and GPP at the seasonal scale. The seasonal variation of processes such as photosynthetic light absorption, CET, adjustments in relative absorption cross-sections of PSII and PSI, alternative sinks, PSI fluorescence contribution, photorespiration, photosystem connectivity, and the thermodynamic effect of temperature need to be characterized in order to understand and mechanistically model the seasonal link between ChlF and photosynthesis.
- (ii) Linking PAM and SIF fluorescence at the seasonal scale. Combined measurements of PAM and spectrally resolved ChlF are needed at the seasonal and leaf scales to elucidate how the methodology affects the information content of the signal.
- (iii) Characterizing the vertical and genetic variation in physiological traits. The variation in factors listed in (i) and (ii) within a plant canopy and across species still needs to be characterized fully in order to identify possible generic functions that could be implemented when up-scaling from the leaf to the canopy and landscape levels.

Acknowledgements

This work has been supported by the Academy of Finland [grant nos 1138884 and 272041 to AP-C and 259075 to ET], and the University of Helsinki (grant no 490116). This review idea originated from discussions during a Workshop organized by the Keck Institute for Space Studies 'New Methods for Measurements of Photosynthesis from Space'. The availability of results from ongoing preparatory studies for the ESA FLEX mission is acknowledged. We thank Professor Kari Heliövaara for help in preparing Figs 2, 4, and 7.

Appendix 1. Interaction between PSII connectivity, fraction of open reaction centres q , and parameters q_L and q_P in the presence of NPQ. An analysis based on equations derived by Lavergne and Trissl, (1995)

Adaptation of the fluorescence yield equation as a function of q for a connected units model

We define a system with two populations of PSII units, open (PSII_{open}) and closed (PSII_{closed}). We assume rapid energy equilibration between peripheral antenna, core, and reaction centre (single pool model). We define ‘ q ’ as the fraction of PSII units with an open reaction centre. k_{LII} is the sum of rate constants of all non-photochemical quenching processes operating in the photosynthetic antenna, namely constitutive thermal dissipation, fluorescence, and regulated thermal energy dissipation (NPQ), $k_{LII}=k_D+k_F+k_{NPQ}$. We assume that thermal dissipation by open reaction centres, intersystem crossing, photobleaching, and spillover are insignificant and are embedded in k_D . Finally, k_{PSII} is the intrinsic rate constant of photochemistry in open reaction centres, and k_{CII} is the rate constant of energy transfer between photosynthetic units (Fig. A1).

According to the above system, three different connectivity models can be defined (Lavergne and Trissl, 1995): (i) a lake model, when k_{CII} tends to infinity; (ii) a separate units model, when k_{CII} tends to zero; and (iii) a connected units model when $0 < k_{CII} < \infty$. We then redefine the parameters J , B , and C derived for a connected units model by Lavergne and Trissl (1995) using our nomenclature, where $k_{PSII}=\alpha_P$ (therein), $k_{LII}=k_I$ (therein), $k_{CII}=k_{UU}$ (therein), $k_{RCII}=\beta$ (therein), and $k_F=k_{rad}$ (therein). Similarly, the rate constants k_{20} and k_{30} in Lavergne and Trissl (1995) are reformulated as (note that α_d , the rate constant of thermal dissipation by open centres, is assumed to be zero):

$$k_{20} = k_{PSII} + k_L \quad (A1)$$

$$k_{30} = k_{RCII} + k_L \quad (A2)$$

and the factors J , B , and C in Lavergne and Trissl (1995) become:

$$J = \frac{k_{CII} (k_{20} - k_{30})}{k_{30} (k_{CII} + k_{20})} = \frac{k_{CII} (k_{PSII} - k_{RCII})}{(k_{RCII} + k_{LII})(k_{CII} + k_{PSII} + k_{LII})} \quad (A3)$$

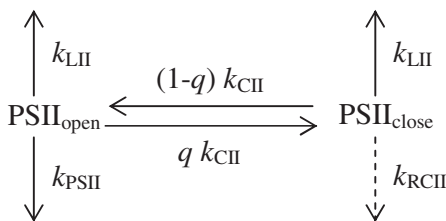


Figure A1. Schematic diagram indicating the main energy pathways in a system with open and closed populations of PSII units.

$$B = \frac{k_F}{k_{30}} = \frac{k_F}{k_{RCII} + k_{LII}} \quad (A4)$$

$$C = \frac{k_F (k_{30} - k_{20})}{k_{30} (k_{CII} + k_{20})} = - \frac{k_F (k_{PSII} - k_{RCII})}{(k_{RCII} + k_{LII})(k_{CII} + k_{PSII} + k_{LII})} \quad (A5)$$

Subsequently, using Equation 6b in Lavergne and Trissl (1995) to express the fluorescence yield as a function of q , we obtain:

$$\Phi F(q) = \frac{B + Cq}{1 + Jq} = \frac{\frac{k_F}{k_{RCII} + k_{LII}} - \frac{k_F (k_{PSII} - k_{RCII})}{(k_{RCII} + k_{LII})(k_{CII} + k_{PSII} + k_{LII})} q}{1 + \frac{k_{CII} (k_{PSII} - k_{RCII})}{(k_{RCII} + k_{LII})(k_{CII} + k_{PSII} + k_{LII})} q} \quad (A6)$$

and rearranging,

$$\begin{aligned} \Phi F(q) &= \frac{\frac{k_F}{k_{RCII} + k_{LII}} \left(1 - \frac{k_{PSII} - k_{RCII}}{k_{CII} + k_{PSII} + k_{LII}} q \right)}{\frac{k_{RCII} + k_{LII}}{k_{RCII} + k_{LII}} + \frac{k_{CII} (k_{PSII} - k_{RCII})}{(k_{RCII} + k_{LII})(k_{CII} + k_{PSII} + k_{LII})} q} = \\ &= \frac{k_F \left(1 - \frac{k_{PSII} - k_{RCII}}{k_{CII} + k_{PSII} + k_{LII}} q \right)}{k_{RCII} + k_{LII} + \frac{k_{PSII} - k_{RCII}}{k_{CII} + k_{PSII} + k_{LII}} q k_{CII}} \end{aligned} \quad (A7)$$

Finally, since the rate constant of thermal energy dissipation by closed PSII centres (with P680⁺) (k_{RCII}) is only relevant for the interpretation of fluorescence data obtained with intense laser sources, we can assume that k_{RCII} equals zero and Equation A7 becomes:

$$\Phi F(q) = \frac{k_F \left(1 - \frac{k_{PSII}}{k_{CII} + k_{PSII} + k_{LII}} q \right)}{k_{LII} + \frac{k_{PSII}}{k_{CII} + k_{PSII} + k_{LII}} q k_{CII}} \quad (A8)$$

Equation A8 was used to estimate F and F_M in Fig. 5, using parameter values reported in Lavergne and Trissl (1995) (Table 1A), and adjusting k_{CII} and k_{LII} to represent different levels of connectivity and NPQ, respectively. For example, $k_{LII}=0.3 \text{ ns}^{-1}$ (NPQ=0), but becomes 0.6 ns^{-1} or 0.9 ns^{-1} if NPQ=1 or 2, respectively, as $\text{NPQ}=k_{NPQ}/(k_F+k_D)$.

Table 1A. Parameter values

Parameter	Correspondence in Lavergne and Trissl	Value (ns ⁻¹)
k_{PSII}	α_P	2.654
k_F	k_{rad}	0.056
k_{LII}	k_I	0.300

Demonstration of q_L and q_P

When k_{CH} approaches infinity (i.e. a lake model), Equation A8 becomes:

$$\Phi F(q)_{\text{Lake}} = \frac{k_F}{k_{LII} + q k_{PSII}} = \frac{k_F}{k_D + k_F + k_{NPQ} + q k_{PSII}} \quad (\text{A9})$$

which is equivalent to Equation 7, demonstrating that q_L is equal to q when connectivity approaches infinity. Conversely, if k_{CH} approaches zero (separate units model), and given that the sum of all quantum yields is by definition equal to unity, then

$$1 - \frac{k_{PSII}}{k_{PSII} + k_{LII}} = \frac{k_{LII}}{k_{PSII} + k_{LII}}, \quad (\text{A10})$$

and we can express Equation A8 as:

$$\begin{aligned} \Phi F(q)_{\text{SeparateUnits}} &= \frac{k_F \left(1 - \frac{k_{PSII}}{k_{PSII} + k_{LII}} q \right)}{k_{LII}} = \\ &= \frac{k_F}{k_{LII}} - \frac{k_F k_{PSII} q}{k_{LII} (k_{PSII} + k_{LII})} = \\ &= (1-q) \frac{k_F}{k_{LII}} + q \frac{k_F}{k_{LII}} - \frac{k_F k_{PSII} q}{k_{LII} (k_{PSII} + k_{LII})} = \\ &= (1-q) \frac{k_F}{k_{LII}} + q \left(\frac{k_F}{k_{LII}} - \frac{k_F k_{PSII}}{k_{LII} (k_{PSII} + k_{LII})} \right) = \\ &= (1-q) \frac{k_F}{k_{LII}} + q \frac{k_F}{k_{LII}} \left(1 - \frac{k_{PSII}}{k_{PSII} + k_{LII}} \right) = \\ &= (1-q) \frac{k_F}{k_{LII}} + q \frac{k_F}{k_{LII}} \frac{k_{LII}}{k_{PSII} + k_{LII}} = \\ &= (1-q) \frac{k_F}{k_{LII}} + q \frac{k_F}{k_{PSII} + k_{LII}} \end{aligned} \quad (\text{A11})$$

which is equivalent to the separate units formulation of q , that corresponds to the parameter q_P (Genty *et al.* 1989). The performance of q_L and q_P under intermediate connectivity scenarios and in the presence of NPQ is assessed in Fig. 5.

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