

Experimental in vivo measurements of light emission in plants: a perspective dedicated to David Walker

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Abstract This review is dedicated to David Walker (1928–2012), a pioneer in the field of photosynthesis and chlorophyll fluorescence. We begin this review by presenting the history of light emission studies, from the ancient times. Light emission from plants is of several kinds: prompt fluorescence (PF), delayed fluorescence (DF), thermoluminescence, and phosphorescence. In this article, we focus on PF and DF. Chlorophyll *a* fluorescence measurements have been used for more than 80 years to study photosynthesis, particularly photosystem II (PSII) since 1961. This technique has become a regular trusted probe in agricultural and biological research. Many measured and calculated parameters are good biomarkers or

indicators of plant tolerance to different abiotic and biotic stressors. This would never have been possible without the rapid development of new fluorometers. To date, most of these instruments are based mainly on two different operational principles for measuring variable chlorophyll *a* fluorescence: (1) a PF signal produced following a pulse-amplitude-modulated excitation and (2) a PF signal emitted during a strong continuous actinic excitation. In addition to fluorometers, other instruments have been developed to measure additional signals, such as DF, originating from PSII, and light-induced absorbance changes due to the photooxidation of P700, from PSI, measured as the absorption decrease (photobleaching) at about 705 nm, or

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increase at 820 nm. In this review, the technical and theoretical basis of newly developed instruments, allowing for simultaneous measurement of the PF and the DF as well as other parameters is discussed. Special emphasis has been given to a description of comparative measurements on PF and DF. However, DF has been discussed in greater details, since it is much less used and less known than PF, but has a great potential to provide useful qualitative new information on the back reactions of PSII electron transfer. A review concerning the history of fluorometers is also presented.

Keywords Delayed fluorescence · Fluorometers · Photosystem II · Prompt fluorescence

Dedication

This historical and educational review is dedicated to a pioneer in the field of photosynthesis and chlorophyll fluorescence: David Walker (see a photograph). For a Tribute to David, see Edwards and Heber (2012). We note that the first four oxygen chambers of Hansatech Instruments were named after David Walker, DW (http://www.hansatechinstruments.com/electrode_chambers.htm). His contributions are enormous, but, in the context of this review, we refer the readers to the following: for parallel measurements on oxygen evolution and chlorophyll *a* fluorescence, see Delieu and Walker (1983), Walker and Osmond (1986), and Walker (1987); for relationship of chlorophyll fluorescence with the onset of carbon fixation, see Walker (1981), and for simultaneous measurement of oscillations in photosynthesis and chlorophyll fluorescence, that is due to cycling of ATP and NADPH, see Walker et al. (1983).

Introduction

History of light emission

Beniamino Barbieri, with the help of David Jameson, has provided a nice history, which deals with discoveries on light emission in living systems. It is available at <http://www.fluorescence-foundation.org/lectures/madrid2010/lecture1.pdf>. (It is best to copy and paste this address, and other web addresses in this review, on the web browser; also see <http://www.iss.com>.) We present here a summary of the early history of light emission in Nature, based on this presentation (accessed on June 12, 2012). On the other hand, E. Newton Harvey (1957) has also described the history of light emission starting from the earliest times (BCE) and ending in the last decades of the nineteenth century; this book is available online at http://www.archive.org/stream/historyoflumines00harv/historyoflumines00harv_djvu.txt; it has included history of all sorts of light emission, many being very dim indeed: e.g., glow of phosphorus, chemiluminescence; phosphorescence of certain solids after they are exposed to sunlight, or to X-rays, or to electron beams; the *aurora borealis*, as well as electroluminescence of gases; and triboluminescence of crystals when they are rubbed or even broken. Discussion of light emission also included bioluminescence from fireflies and glow worms, often described as “burning of the sea,” and the light that is emitted from fungus attached to decaying tree trunks. It even included light from bacteria on dead flesh or fish. We shall not be talking about these types of light emission in this review, but we will focus on light emission, particularly from chlorophyll *a* in photosynthetic organisms; as a prelude, we will present below the early history of research on light emission from plants, as well as from other sources.



A photograph of David Walker (third from the left, wearing a blue jacket and holding a small package) with others at the 12th International Photosynthesis Congress, held in 2001 in Brisbane, Australia (Govindjee and Yoo 2007)

We begin with 1565 when Nicolás Monardes, a Spanish physician and botanist, published his “The Historia medicinal de las cosas que se traen de nuestras Indias Occidentales”; he observed a “bluish opalescence” in the water infusion from the wood of a small Mexican tree. In 1574, a Flemish botanist Charles de L’Écluse (1526–1609) provided a Latin translation of Monardes’ work, from where we find that the name of the wood, used by Monardes, is *Lignum nephriticum* (kidney wood); it was very popular then because of its use in treating kidney diseases. Interestingly, a 1577 translation by an Englishman John Frampton, reads “white woodde which gives a blewe color” when placed in water that was good “for them that doeth not pisse liberally and for the pains of the Raines of the stone.” Athanasius Kircher (1601–1608), a German Jesuit priest, wrote, in his 1646 book “Ars Magna Lucis et Umbrae” “Light passing through an aqueous infusion of this wood appeared more yellow while light reflected from the solution appeared blue.” Robert Boyle (1664) investigated this system more precisely, and found that the wood would lose the ability to color the water after several infusions; thus, he concluded that there must have been some “essential salt” in the wood that gave the blue color. Further, acid abolished the color and alkali restored it—a pH effect. In 1915, W.E. Safford showed that the Mexican *Lignum nephriticum* is taxonomically *Eynsehardtia polystachia*. (For further history on the color and the chromophores that may have produced this blue light, see the web site at <http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorointrohome.html>).

Then, there was the discovery of light from a stone, this one from Bologna. A Bolognian shoemaker Vincenzo Casciarolo, who was dreaming of producing gold, discovered, in 1603, that a stone (later named *laparis solaris*) emitted purple-blue light in the dark, after it had been baked. The famous Galileo Galilei (1612) got in the act and described the emission of light from the Bolognian stone as a sort of phosphorescence:

It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth.

The history of light emission from chlorophyll begins with David Brewster (1834), a Scottish preacher, who described that when a beam of bright sunlight passed through an alcoholic extract of laurel leaves, brilliant red color was observed from the side (see Govindjee 1995). Brewster must have used his eyes to make this observation. This solution must have contained chlorophyll (Chl) that had been so named as such by Pelletier and Caventou (1818). Soon thereafter, John Herschel (1845) made the first observation, with his eyes, of fluorescence, a beautiful blue light, from quinine sulfate (a component of what is in

“tonic water”)—he termed this phenomenon “epipolic dispersion.”

His paper was titled:

On a case of superficial colour presented by a homogeneous liquid internally colourless. By Sir John Frederick William Herschel, Bart., K.H., F.R.S. (Received January 28, 1845—Read February 13, 1845)

The Bolognian stone, mentioned above, has been known to be impure barium sulfide. It is interesting to note that Edmond Becquerel (in 1842) reported that excitation of calcium sulfate by ultraviolet (UV) light produced bluish emission. Becquerel noted that the emission occurs at a wavelength longer than that of the incident light. This phenomenon became well known when the British mathematician Sir George G. Stokes discovered that emission spectra are shifted to longer wavelengths than the absorption spectra; this shift has been named the “Stokes shift” in honor of its discoverer. (For a biography of Stokes, see <http://www.giffordlectures.org/Author.asp?AuthorID=160>.) When sunlight filtered through a blue-glass from a church window (exciting light, <400 nm) impinged on a solution of quinine, Stokes saw, with his eyes, blue light through a yellowish glass of wine (transmitting >400 nm) (See Lakowicz 1999). Stokes had later used a prism to get different wavelengths of light to illuminate a solution of quinine. No emission was obtained until the solution was placed in the UV region of the spectrum. This observation led Stokes to declare that fluorescence is of a longer wavelength than the exciting light, which led to this difference between absorption and emission being called the Stokes shift, as mentioned above.

We also note that it was Stokes, who, in 1852, coined the term *fluorescence* for this phenomenon, while making reference to the blue-white fluorescent mineral fluorite (fluorspar). Also, Stokes was, perhaps, the first one to have observed both phycobilin and Chl *a* fluorescence in fresh red algae (Askenasy 1867). In addition, he was the first to suggest, in 1864, that fluorescence should be used as an analytical tool, when he gave his lecture “*On the application of the optical properties to detection and discrimination of organic substances*”...

Stokes published his massive treatise “On the Change of Refrangibility of Light”; he initially had used the term “dispersive reflection” to describe light emission in quinine sulfate.

One of his papers had the following title:

On the change of Refrangibility of light. By G.G. Stokes, M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge. Received May 11—Read May 27. 1852

“Luminescenz” implies light, and was first used in 1888 by Eilhard Wiedemann, a German physicist and a historian of science; this term described “*all those phenomena of light, which are not solely conditioned by the rise in temperature.*” It is well known that all liquids and solids emit radiation of shorter wavelengths as they are heated above absolute zero: we can watch the material become red hot and then white hot. This is described as “hot light,” and it has a different physical basis than that of luminescence, which is “cold light.” In our everyday life, we see the light from the Sun; we also use flash-light (run on batteries); we light candles on festive occasions; and then we use “oil lamps, gas burners, and electric light bulbs as well as fluorescent tubes”. For further discussion, see Harvey (1957).

Fluorescence

Fluorescence is a member of the ubiquitous luminescence family of processes in which chromophore (pigment-bearing) molecules emit light from electronically excited singlet states produced either by a physical (for example, absorption of light, sound or pressure), mechanical (friction), or a chemical mechanism. Generation of luminescence through excitation of a molecule by UV or visible light is a phenomenon termed photoluminescence, which is formally divided into two categories, fluorescence and phosphorescence, depending upon the electronic configuration of the excited state and the emission pathway. Fluorescence is the property of some atoms and molecules that absorb light at a particular wavelength and subsequently emit light, usually at longer wavelengths, after a brief interval. Lifetime of this fluorescence gives information on the rate constant of this process (see e.g., Noomarm and Clegg 2009). The process of phosphorescence occurs in a manner similar to fluorescence, but with a much longer excited state lifetime (for related information, see <http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorhome.html>). Fluorescence originates from de-excitation of the lowest singlet excited states, whereas phosphorescence originates in triplet states (see discussion in Lakowicz 1983). In this review, we shall focus on fluorescence and delayed fluorescence (DF) (see below). For phosphorescence in photosynthetic systems, see e.g., Krasnovsky (1982) and Neverov et al. (2011). For an understanding of fluorescence, see Valeur and Berberan-Santos (2012).

As mentioned above, we include in this review prompt fluorescence, for short PF (that is light emission when an electron in the first excited singlet state drops down to the ground state) and DF (also known as delayed light emission, DLE), when the first singlet excited state is reached via recombination of charges, not by light absorption. For an earlier review that deals with both PF and DF, see

Govindjee and Jursinic (1979); for DF, see Lavorel (1975); and for PF, see Butler (1966).

PF from chlorophyll *a*

We have already alluded to the discoveries of Brewster and Stokes (see above). Müller (1874) provided one of the earliest clues of relationship of fluorescence with photosynthesis although his experiments were not performed with good controls and he had erroneous ideas of the basic concepts of light; he had, however, noticed that a living green leaf had a much weaker red Chl fluorescence than a Chl solution (Govindjee 1995). Kautsky and Hirsch (1931), using their own eyes, observed Chl *a* fluorescence to rise rapidly to a maximum, then decline, and finally reach a steady level, all within a matter of minutes. The rising portion of the curve was considered to reflect the primary photochemical reaction of photosynthesis, as it was unaffected by temperature (0 and 30 °C). The decline in the fluorescence curve appeared to be inversely correlated with the increase in the rate of CO₂ assimilation, measured earlier by Warburg (1920); this suggested to Kautsky and Hirsch (1931) that more chemical energy is produced from photons when less Chl fluorescence is seen (*complementary relationship*). This study was followed by Chl fluorescence transient measurements on photosynthetic samples with many available instruments, by many investigators, showing many inflection points, which were labeled A, B, C, D, E, or D1, M1, D2, M2 (reviewed by Rabinowitch 1951). The current nomenclature of the fast (up to 1–2 s) Chl *a* fluorescence transient is OJIPS (where O is the origin, the minimum level; J and I are inflections; P is the peak, and S is the steady state) (see e.g., Strasser and Govindjee 1992; Strasser et al. 1995, 2004, 2010; Stirbet and Govindjee 2011, 2012). (See Fig. 1 for OJIPSMT chlorophyll *a* fluorescence transient in a pea leaf.) There is a history to this nomenclature. The O I(D)P nomenclature for the fast transient, or the first wave, lasting up to a second, is based on those by Lavorel (1959, 1963): O (origin; minimum) → P (peak), and by Munday and Govindjee (1969a, b): O → I → D → P (D was introduced for a dip). The slow transient, or the second wave, following P, lasts up to several minutes. Papageorgiou and Govindjee (1968a, b) called it SMT, where S is the semi-steady state, M is the maximum, and T is the terminal steady state. Sometimes, there are several additional waves in between; they have been labeled as, e.g., S1, M1, S2, M2, and T (Yamagishi et al. 1978); this nomenclature was partly based on a terminology of Bannister and Rice (1968); On the other hand, Neubauer and Schreiber (1987) and Schreiber and Neubauer (1987) had used O–I₁–I₂–P for the fast transient when they had seen two instead of one inflection between O and P; the O–I₁–I₂–P nomenclature

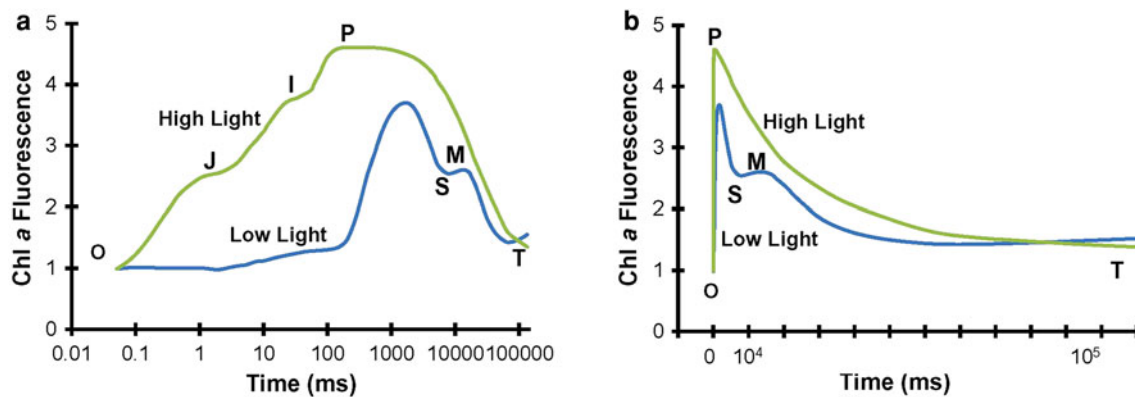


Fig. 1 Typical chlorophyll *a* fluorescence induction transients (Kautsky curves), at two different excitation light intensities. Sample: a 20 min dark-adapted pea leaf. *Right* on a logarithmic time scale, *Left* on a linear time scale. Wavelength of excitation: 650 nm. Excitation light intensity for curves labeled *low light* was ~ 30 mol photons $\text{m}^{-2} \text{s}^{-1}$ at the leaf surface; for high light, $\sim 3,000$ mol photons $\text{m}^{-2} \text{s}^{-1}$ at the leaf surface. In the O–J–I–P–S–M–T nomenclature, O stands for the origin (minimum), J and I are intermediate inflections, P is for peak, S is for semi-steady state, M is for maximum, and T for terminal steady state (also see text). Fluorescence values are expressed as F/F_0 , where F_0 is the initial fluorescence (at 50 μs) and F is fluorescence at any other time; thus, it is in relative or

was replaced by the OJIP nomenclature of Strasser and Govindjee (1992) for simplicity. For further discussion, see Schreiber and Krieger (1996). Beside the clearly visible steps in the OJIP fast fluorescence rise, several inflections can be revealed under specific conditions, or in certain photosynthetic organisms, directly during the O to P transient or from the difference kinetics of two different curves; the nomenclature of these fluorescence bands between F_0 ($=O$) and F_p ($=P$) (from shorter to longer times) as used by Strasser et al. (2007) is (in reverse alphabetical order: L to F): O–(L–K–)J–I–(H–G–) $F_m = P$; in addition, F_t has been regularly used for fluorescence at time “*t*.” In this review, we do not discuss further the L, K, H, and G points. (For application of fluorescence to stress conditions, there are many papers, but for a simple and a quick application of fluorescence to stress conditions, see Lichtenthaler and Rinderle 1988.)

MacAlister and Myers (1940) obtained the first quantitative complementary relationship between fluorescence and photosynthesis (i.e., CO_2 assimilation or O_2 evolution). However, it was Delosme et al. (1959) who showed a parallel relationship between fluorescence and photosynthesis (i.e., O_2 evolution) during the fast O to I fluorescence rise! A major concept was provided by Duysens and Sweers (1963) who proposed the “*Q*” hypothesis, i.e., fluorescence being low when *Q* (now called Q_A) is in the oxidized state, and fluorescence being high when Q_A is reduced to Q_A^- . Further, Papageorgiou and Govindjee (1968a, b) and Mohanty et al. (1971) showed a parallel increase in fluorescence and O_2

arbitrary units. The initial low fluorescence intensity is interpreted as being due to quenching of fluorescence by highly efficient PSII photochemistry. The rise in fluorescence is due mainly to reduction of Q_A , an electron acceptor of PSII (for recent reviews, see Stirbet and Govindjee 2011, 2012). The slow fluorescence transient (P–S–M–T) is due to several reasons including (i) reoxidation of reduced Q_A , (ii) quenching by transmembrane ΔpH , and (iii) transition from high fluorescent state I to low fluorescent state II (see a review by Papageorgiou and Govindjee 2011). Source of the original figure: Strasser et al. (1995); modified by Alaka Srivastava, and as published by Stirbet and Govindjee (2011)

evolution during the slow S to M phase, and constancy of O_2 evolution during the MT decline. Thus, it is clear that the anti-parallel relationship is observed only under certain experimental conditions. We know very well that de-excitation of an excited state has at least four pathways: (1) photochemistry, (2) fluorescence, (3) heat, and (4) excitation energy migration to neighboring pigment complexes (cf. Govindjee 2004). For a review on Chl *a* fluorescence induction, see Lazar (1999). Of special importance is the last process. As just one example, Strasser and Butler (1977a, b) showed that excitation energy transfer from pigment system II (PSII) to pigment system I (PSI) led to an appreciable amount (over 50 %) of PSI activity (P700 photooxidation) at 77 K, when light was absorbed originally in PSII.

If the quality or quantity of light suddenly changes, then the redox states of the different systems in the whole photosynthetic electron transport chain between water and NADP also change (see Lawlor 2001). The system adapts to the new conditions by conformational changes and reaches again the steady-state conditions.

Bannister and Rice (1968) and Papageorgiou and Govindjee (1968a, b) presented parallel measurements on time course of oxygen evolution and chlorophyll fluorescence in the green alga *Chlorella* and in a cyanobacterium *Anacystis*. Further, Walker et al. (1983) presented their parallel measurements on the oscillations of oxygen evolution and of Chl fluorescence in spinach leaf pieces. They observed a clear anti-parallel relationship between $d[\text{O}_2]/dt$ and fluorescence curves. The oscillation of oxygen evolution,

plotted versus the oscillation of fluorescence emission, exhibits a function with a spiral shape tending to reach at a steady specific point, called an “attraction point;” this means that fluorescence and oxygen evolution have similar trends (e.g., rise and decays or oscillations), however, with different changes in the rate constants (Strasser 1985, 1986). An anti-parallel relationship between fluorescence and photochemistry is possible only if the heat loss is negligible and constant. For further historical discussion, see reviews by Govindjee (1995, 2004).

The light that is emitted at the time of the de-excitation of the first excited state of Chl contains two components: PF and DF; in general, the DF component is only a very small part of this emission (however, see Klimov et al. 1978). The PF emission is practically extinguished about 5 ns after the light is switched off (see reviews: Jursinic 1986; Krause and Weis 1991); its intensity decays in a polyphasic manner, with characteristic lifetimes that range from several ps to 2 ns (see e.g., Miloslavina et al. 2006). Light emission by antennae Chl molecules after the PF has decayed is called DF. DF decays in the dark, also in a polyphasic manner, and has components in very different time domains: in the *nanoseconds* (Christen et al. 2000), *microseconds* (Jursinic and Govindjee 1977; Jursinic et al. 1978; Wong et al. 1978; Christen et al. 1998; Mimuro et al. 2007; Buchta et al. 2008; Kocsis et al. 2010) *milliseconds* (Hipkins and Barber 1974; Barber and Neumann 1974; Zaharieva and Goltsev 2003; Goltsev et al. 2005; Buchta et al. 2007; Kocsis et al. 2010), *seconds* (Rutherford et al. 1984; Hideg et al. 1991; Katsumata et al. 2008), and even in the *minute-to-hour* time range (Hideg et al. 1990). However, in the latter time range, an involvement of lipid peroxidation in Chl photon emission could also take place. In the latter case, photons are emitted by Chls as a consequence of lipid peroxidation processes initiated by reactive oxygen species (Hideg et al. 1991). Recently, a detailed mechanism of Chl photon emission has been described under in vivo condition (Prasad and Pospíšil 2011). These authors demonstrated that the excitation energy transfer from triplet excited carbonyl and singlet oxygen formed during lipid peroxidation results in the formation of excited Chl, the de-excitation of which leads to the emission in the red region of the spectrum. We will not describe here the mechanisms of PF, but we refer the readers to chapters in books on PF (Govindjee et al. 1986; Papageorgiou and Govindjee 2004). However, we will discuss DF in some details since it is much less used and known than PF.

DF from chlorophyll *a*

The DF (also called DLE), is a lower intensity longer lived light emission than PF. Strehler and Arnold (1951) (also

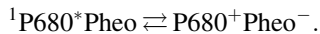
see Strehler 1951) discovered it rather accidentally, as a very weak light emission, while they were attempting to measure the production of ATP in the green alga *Chlorella* (for historical details, see Strehler 1996). This DF must originate in the de-excitation of excited Chl *a* since it has an emission spectrum almost identical to that of Chl *a* fluorescence (Arnold and Davidson 1954; Arnold and Thompson 1956; Lavorel 1969; Clayton 1969; Sonneveld et al. 1980b; Grabolle and Dau 2005). The similarity between the emission spectra of DF and PF shows that in both cases the photon release is a result of the radiative deactivation of the singlet excited state of Chl *a* in the PSII antenna (Krause and Weis 1991; Lang and Lichtenthaler 1991). Indeed, it is well established that DF in plants, algae, and cyanobacteria originates mainly in PSII, since it is absent in algal mutants that lack PSII (Bertsch et al. 1967; Lavorel 1969; Haug et al. 1972; Bennoun and Béal 1997; Turzó et al. 1998). Further: (1) the action spectra for PSII activity and DF are almost identical (Arnold and Thompson 1956; Lavorel 1969) and (2) there is a 60- to 90-fold higher level of DF in PSII than in PSI-enriched particles (Lurie et al. 1972; Vernon et al. 1972; Itoh and Murata 1973; Gasanov and Govindjee 1974). Although PSI does have DF (Shuvalov 1976), it has significantly much lower yield than that from PSII.

Various aspects of DF have been discussed earlier by Arnold (1965, 1977), Mar and Govindjee (1971), Fleischman and Mayne (1973), Mar and Roy (1974), Lavorel (1975), Malkin (1977, 1979), Jursinic (1977, 1986), Ames and Van Gorkom (1978), and Govindjee and Jursinic (1979).

Mechanism of DF

DF, observed in plants, algae, and cyanobacteria, is intimately connected with the primary processes of light energy conversion in the reaction center of PSII, as mentioned above. Since the detailed description of exciton trapping mechanisms has been possible only recently after the development of ultrafast instrumentation, this topic is still a matter of debate (for different opinions, see Barber 2002; Durrant et al. 1995; Shelaev et al. 2011; Holzwarth et al. 2006; Romero et al. 2012); we will not discuss its ramifications here. According to the reversible radical pair (RRP) hypothesis (see e.g., Schatz et al. 1988): (1) there exists a fast equilibrium between the excited state of the primary PSII electron donor $^1\text{P680}^*$ and Chl antenna of PSII and (2) the radical pair $\text{P680}^+\text{Pheo}^-$ can recombine if the charge separation in the couple is not stabilized through fast reoxidation of reduced pheophytin by the first (plasto) quinone electron acceptor Q_A (for a general review on PSII, see Govindjee et al. 2010). The DF quanta are emitted from the excited antenna Chl formed as a result of

reversal of charge separation, followed by a fast excitation energy transfer from $^1\text{P680}^*$ to Chl antenna (Dau and Sauer 1996; Grabolle and Dau 2005):



This DF emission with a lifetime of 2–4 ns is the fastest decaying component of DF, but it is difficult to be quantified, since it cannot be easily separated from the PF. Moreover, there are theories (e.g., Klimov et al. 1978) that assume that this ns DF contributes significantly to the variable fluorescence, which generally is considered part of PF.

The DF dark decay is determined by a decrease in the number of charge couples (precursors of excited states of $^1\text{P680}^*$ and, thus, of emitted DF quanta) or by changes in the DF quantum yield. All redox reactions with the charge couple—oxidation or reduction by neighboring carriers or back electron transport reaction inside the couple—result in the elimination of the DF precursor. Usually, the back reactions are several orders of magnitude slower than forward reactions and their contribution to the DF decay rate could be neglected (Lavorel 1975). When forward electron transfer is stopped (e.g., when PQ pool is fully reduced, or in the presence of PSII herbicides), then the back reactions determine the DF decay.

The processes that determine the kinetics of DF dark relaxation are classified into three groups (cf. Lavorel 1975; Goltsev et al. 2009):

- (1) The “leakage” type reactions—when the decrease in DF precursors is mainly the result of the disappearance of the negative or positive charges from the radical pair $\text{P680}^+\text{Pheo}^-$, as e.g., due to: (a) reoxidation of Pheo^- by Q_A or (b) reduction of P680^+ by the electron donor Z (i.e., Y_Z). This is the main mechanism of DF decay in micro- and sub-millisecond time range.
- (2) The “deactivation” type reactions—when DF precursors decrease due to redox reactions within the charge pair (i.e., by recombination of charges within $\text{P680}^+\text{Pheo}^-$) (cf. Lavorel 1975; Klimov et al. 1978). However, a small part of this recombination reaction can lead to the formation of the excited state of P680 ($^1\text{P680}^*$), and thus to DF. The “deactivation” of DF precursors ($\text{P680}^+Q_A^-$, $Z^+\text{P680}Q_A^-$, or $S_2Z\text{P680}Q_AQ_B^-$) through backward electron transport reactions, followed by charge recombination, contributes to the slower DF components (milliseconds and longer). In both the “leakage” and the “deactivation” cases, the DF intensity decreases because of the disappearance of the separated charge couples.
- (3) The “de-energization” type reactions—which affect the kinetics of DF dark decay by modifying the rate constant of recombination of the charge couples, and

correspondingly the DF quantum yield. These processes are related to the dark deactivation of the energized state of the thylakoid membrane (proton trans-thylakoid gradient, ΔpH and membrane potential, $\Delta\psi$).

When the reactions that determine the dark decay are of the first order (as is the case for the leakage type DF), the DF relaxation curve can be described as a sum of exponential functions:

$$L(t) = \sum_i L_i e^{-t/\tau_i}, \quad (1)$$

where $L(t)$ (L for luminescence) is DF emitted at time t after the light is switched off; L_i is the amplitude of the i th component, and τ_i is its characteristic lifetime. Using this model, the deactivation type of DF can also be described, when the separated charges remain in the same protein complex, as is the case, for example, for the PSII state: $Z^+\text{P680}Q_A^-$, where Z (i.e., Y_Z) is tyrosine-161 on the D1 protein of PSII.

$\text{P680}^+\text{Pheo}^-$ is the only *direct* precursor that recombines and forms the excited state of Chl in PSII reaction center. The other PSII redox states that are the main DF precursors, and, thus, responsible for DF generation are: $\text{P680}^+Q_A^-$, $Z^+Q_A^-$, $Z^+Q_B^-$, and $S_iZ^+Q_B^-$, where Q_B is the second plastoquinone electron acceptor of PSII. All routes for DF generation are the result of backward electron transfer, formation of $\text{P680}^+\text{Pheo}^-$ and their recombination. DF emitted in the microsecond and the millisecond time domain is mostly related to backward electron transfer and recombination of charges in $\text{P680}^+Q_A^-$ and $Z^+Q_A^-$ states of PSII (see Fig. 2 for an energy level diagram that explains DF).

When DF originates from PSII in the $Z^+Q_A^-$ state, the kinetics of its dark decay depends on the rates of the following three redox reactions (see Fig. 2): (a) reoxidation of the reduced acceptor (Q_A^-) by Q_B with a rate constant k_4 ; (b) the reduction of Z^+ (with rate constant k_5) and the transition from state $S_iZ^+Q_A^-$ to state $S_{i+1}Z^+Q_A^-$; and (c) charge recombination between Z^+ and Q_A^- . The last reaction determines the DF decay rate when the direct redox reactions are interrupted by physical or chemical treatments.

Measurements of chlorophyll fluorescence

Fluorometric method

Fluorescence in the blue to the red region of the spectrum occurs, when a molecule absorbs UV–visible photons: light absorption leads to transfer of an electron from the ground state to the excited state of the molecule; as the electron returns to the ground state, the molecule rapidly emits

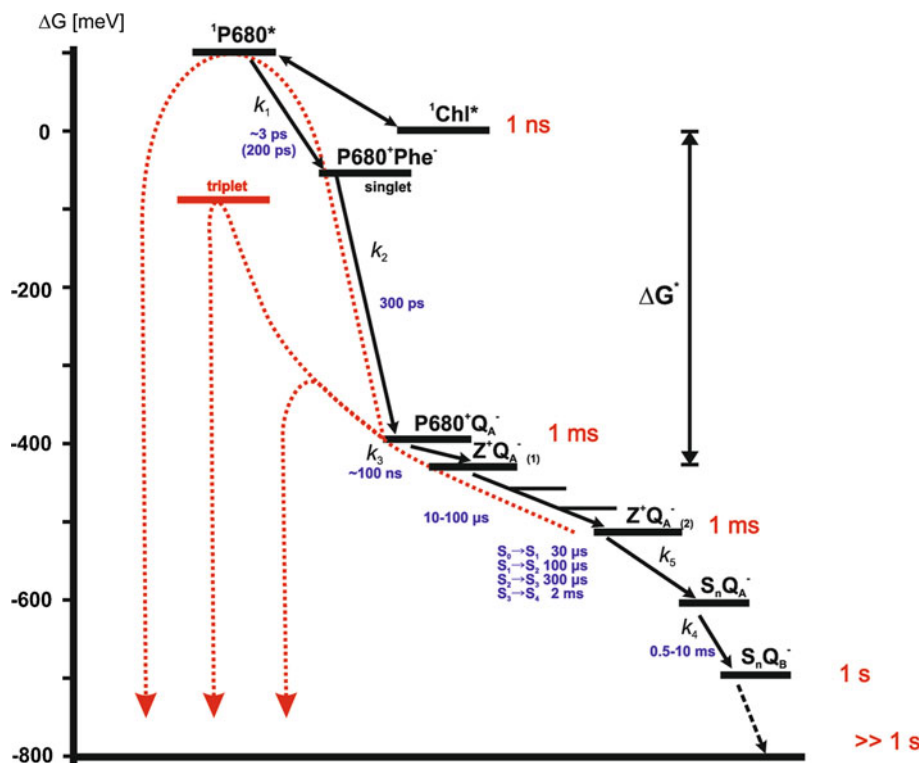


Fig. 2 Energy level diagram for the photosystem (PS) II states participating in DF generation. ΔG values (in meV) (on the left ordinate) indicate estimated Gibbs free-energy levels of PSII redox states participating in DF generation. $^3\text{P680}$ is represented simply by “triplet” in the diagram. For this diagram, the ΔG value of the excited state of antennae chlorophyll (Chl^*) is arbitrarily chosen to be zero. Forward reactions are shown with black arrows; and backward reactions are shown with red dotted lines. K_i s are rate constants of electron transfer (ET) reactions within PSII: k_1 is for primary charge separation in (singlet) excited PSII reaction center chlorophyll; k_2 is for ET from reduced Pheo to Q_A ; k_3 is for ET from the electron donor Z (also called Y_z) to P680^+ ; $\text{P680}^+\text{Pheo}^-$ is the PSII primary radical pair. The rate constants k_3 and k_5 represent ET reactions on the

light: this is the PF (for basics and principles, see Clayton 1971; and see Lakowicz 1983 for details). Measurements of excitation (action) and emission spectra characterize the relationship between absorbed and emitted photons at different wavelengths. It is a precise quantitative analytical technique that is relatively inexpensive and easily mastered. It can also be measured remotely, from a few millimeters with conventional portable fluorometers (Schreiber et al. 1986; Maxwell and Johnson 2000), to several meters (Flexas et al. 2000; Moya et al. 2004), or up to the near-future satellite measurements of passive sun-induced chlorophyll fluorescence (Grace et al. 2007). In addition, it works as an excellent monitoring system over a wide range of time-scales. Therefore, we can study diurnal (Sweeney et al. 1979), as well seasonal acclimation of PSII (Porcar-Castell 2008).

The use of molecular fluorescence for qualitative analysis and semi-quantitative analysis can be traced to the early to mid-1800s, with more accurate quantitative

electron donor side of PSII, and k_4 is for the acceptor side of PSII. The formation of the (initial) state $\text{Z}^+\text{Q}_A(1)^-$ is followed by short- and long-range proton movements (Dau and Zaharieva 2009; Dau et al. 2012), which is accompanied by a decrease in energy (there are intermediate levels before the final state $\text{Z}^+\text{Q}_A(2)^-$ is formed). The numbers in blue are the approximate values of the corresponding characteristic times, i.e., of the reciprocal rate constants for various steps; “ S_n ” represent(s) the so-called S-states of the oxygen evolving complex on the electron donor side of PSII. The values in red are lifetimes of the back reactions. Modified by one of the coauthors (VG), from Grabolle and Dau (2005), Dau and Zaharieva (2009), and Dau et al. (2012)

methods appearing in the 1920s (Hodak et al. 1998). Instrumentation for fluorescence spectroscopy using filters and monochromators for wavelength selection appeared in the 1930s and 1950s, respectively. Although the discovery of phosphorescence preceded that of fluorescence by almost 200 years, qualitative and quantitative applications of molecular phosphorescence did not receive much attention until after the development of fluorescence instrumentation (Valeur 2001; Valeur and Berberan-Santos 2012).

Selected examples

Measurements (e.g., of kinetics, action and emission spectra, depolarization; and lifetimes) of Chl *a* fluorescence, both at physiological and low temperatures, after dark in continuous light have provided critical information on almost every aspect of light absorption and conversion

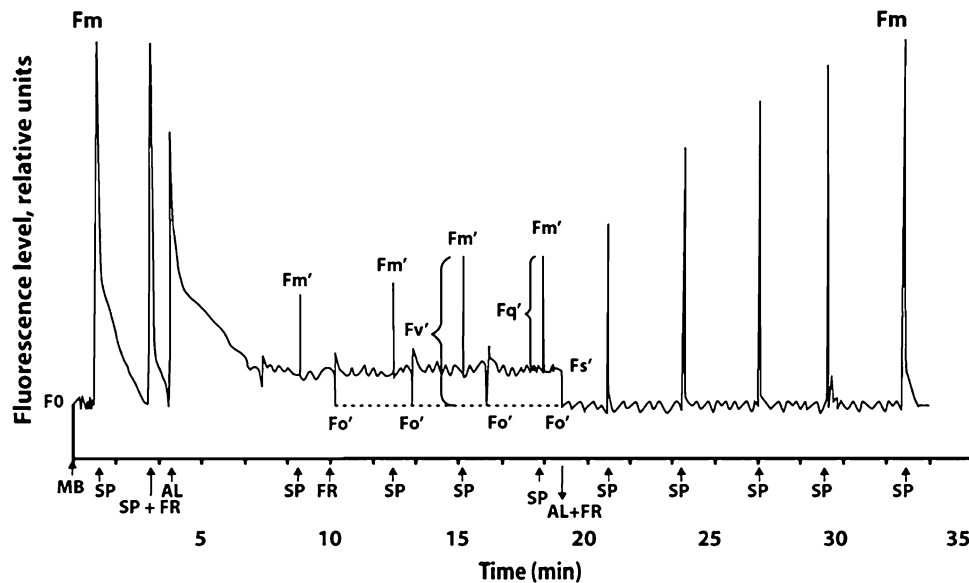


Fig. 3 A schematic tracing of Chl *a* fluorescence measurement using a pulse-amplitude-modulated (PAM) fluorometer (see e.g., Schreiber 2004). A dark-adapted photosynthetic sample was first exposed to a very weak measuring beam (MB) to give the initial fluorescence level F_0 (the “O” level). Then, a saturating light pulse (SP) was applied to obtain the fluorescence maximum F_m , which slowly returns to minimum F_0 . This return can be accelerated by applying a far-red (FR) light, absorbed mostly in photosystem I (PSI) to the sample; this F_0 level is called F_0' , whereas, F_s' refers to steady-state fluorescence in light. Other symbols F_v' and F_q' are defined as $(F_m' - F_0')$, and,

$(F_m' - F_s')$, respectively (see e.g., Baker and Oxborough 2004). After turning on the actinic light (AL), a number of SPs are given to suppress the photochemical quenching and reveal the light-adapted fluorescence maximum $F_m' (< F_m)$. After switching off the AL, there occurs a progressive recovery of the maximum fluorescence level (F_m) obtained after a SP, which reflects the relaxation of the NPQ. The full recovery to the F_m level indicates that no RCIIIs have been irreversibly damaged during the light period. Source of the figure and part of its legend: Henriques (2009)

process that occurs in photosynthesis, with special relevance to our understanding of the (1) excitation energy migration within the antennae and transfer to the reaction centers, (2) energetic connectivity between the antennas and the reaction centers, and (3) primary photochemistry and the secondary electron transport associated with the primary reactions. See reviews on the use of fluorescence technique in photosynthesis studies in books, edited by Govindjee et al. (1986) and by Papageorgiou and Govindjee (2004). For a review on plant leaves, see Henriques (2009); and for reviews on fluorescence transients, see e.g., Lazar (1999); Stirbet and Govindjee (2011, 2012) and Papageorgiou and Govindjee (2011), and references therein. Figure 3 shows pulse-amplitude modulation (PAM) method (see Schreiber 2004) that allows one to obtain information dealing with the measurement and questions related to non-photochemical quenching (NPQ) (Fig. 3).

While Chl *a* fluorescence measurements have been widely used and the field is enormous (Baker 2008; Bussotti et al. 2011a), we list below references to a very small fraction of this literature. Chl fluorescence measurements have proven to be a trusted tool not only for the study of the structure and function of photosynthetic apparatus (Govindjee et al. 1976; Eaton-Rye and

Govindjee 1988a, b; Allakhverdiev et al. 1994; Strasser and Strasser 1995; Bukhov and Carpentier 2000; Bukhov et al. 2001; Antal et al. 2007; Allakhverdiev 2011; Garcia-Mendoza et al. 2011; Matsubara et al. 2011; Brestič et al. 2012; Goltsev et al. 2012), but also in several other areas. These include plant breeding (Baker and Rosenqvist 2004; Kalaji and Guo 2008; Kalaji and Pietkiewicz 2004), seed vigor and seed quality (Jalink et al. 1998; Dell’Aquila et al. 2002; Konstantinova et al. 2002), fruit quality and in controlling the postharvest processing of fruits and vegetables (Merz et al. 1996; Nedbal et al. 2000a). Further, fluorescence is used for the monitoring of plant stress (Kalaji and Nalborczyk 1991; Kalaji and Pietkiewicz 1993; Baker and Oxborough 2000; Nedbal et al. 2000b; Allakhverdiev and Murata 2004; Bussotti 2004; Allakhverdiev et al. 2007a; Ducruet et al. 2007; Van Rensen et al. 2007; Brestič et al. 2010; Yusuf et al. 2010; Živčák et al. 2010; Kalaji et al. 2011a, b, 2012; Kościelniak et al. 2011), of climate change (Ashraf and Harris 2004), of urban conditions (Swoczyna et al. 2010a, b), of environment and pollution (Croisetiere et al. 2001; Bussotti et al. 2005; Kalaji and Łoboda 2007; Romanowska-Duda et al. 2010; Tuba et al. 2010; Bussotti et al. 2011b), of sports field heterogeneity and physiological state (Lejealle et al. 2010; Beard 2002), and of algal blooms and water quality (Gorbunov

et al. 1999; Seppälä et al. 1999; Romanowska-Duda et al. 2005; Antal et al. 2009). This technique is used even in identifying specific nutrient deficiency in plants (www.fluorimetrie.com/ [this site is in French, but English translation is also available]). Recently, a Space Fluorometer (System), produced by NASA researchers, allowed the development of the first-of-its-kind fluorescence map which offers a new view of the world's plants (To reach several useful educational sites at National Aeronautics and Space Administration (NASA), go to the web site at <http://daac.gsfc.nasa.gov/oceancolor/scifocus/oceanColor/warming.shtml>).

In this review, we have attempted to document, for the first time, a wide range of information related to the progress in instrumentation. We also concentrate on the advantages and features of some of the latest available fluorometers and briefly provide relevant technical background.

Tools and instruments to measure fluorescence— history and progress of fluorometers

In this section, we provide for the readers our perspective on the evolution of tools and instruments in a chronological manner.

Observation by the naked eye

We have already mentioned Kautsky and Hirsch's 1931 observations with their naked eyes. Hans W. Kautsky (1891–1966) was a docent (assistant professor) at the Chemisches Institut der Universität in Heidelberg, Germany, when he observed the variable Chl *a* fluorescence in plants. (He was at that time investigating the action and properties of active singlet oxygen in photosensitization processes.) Kautsky and Hirsch (1931) observed an increase in fluorescence intensity, as noted earlier, when dark-adapted plants were illuminated; this observation was published in *Naturwissenschaften* as a one page article titled "New experiments on carbon dioxide assimilation." The time course of Chl *a* fluorescence, *observed with the authors' naked eyes*, was qualitatively correlated with the time course of CO₂ assimilation, published earlier by Otto Warburg (1920) (See Govindjee (1995) for a historical account.) Furthermore, it was Kautsky who had first proposed that the singlet oxygen is a quencher of fluorescence during CO₂ assimilation. His work was long ignored and it was only in 1964 that his research on the role of singlet oxygen in photosynthesis was recognized. The following web site is dedicated to Kautsky's work on fluorescence and other areas: http://www.fluoromatics.com/kautsky_effect.php. Several highly useful websites may be found by going to: <http://www.fluoromatics.com>.

Observations and measurements by laboratory instruments

A detailed history of luminescence has been written by E. Newton Harvey in 1957 (see Harvey 1957). It is available at http://www.archive.org/stream/historyoflumines00harv/historyoflumines00harv_djvu.txt as mentioned earlier. The following description is based on this source.

As described by Harvey (1957), Beccari (1744) had made a device, where he exposed materials to sunlight, and then examined it quickly in the dark; he was able to see phosphorescence that lasted seconds or even tenths of a second. More than 100 years later, Edmond Becquerel (1858) pioneered the construction of the first phosphoroscope that allowed him to measure the decay times of phosphorescence. Then, E. Wiedemann (1888) built a phosphoroscope that shortened the time of the first measurements down to a few microseconds; further, this work provided one of the first observations that suggested that the lifetime of fluorescence of pigments/chromophores was even shorter than that time.

During the early part of the twentieth century, many scientists observed fluorescence in samples under their microscopes; in fact, August Köhler and Carl Reichert initially considered fluorescence as "a nuisance" when they were doing microscopy under UV light. The very first fluorescence microscopes were produced, during 1911–1913, in the laboratories of Otto Heimstädt and Heinrich Lehmann. It was E. Gaviola who designed, in 1926, the very first instrument for measurements of the lifetime of fluorescence; his instrument was based on the principle of phase shift of fluorescence from that of the exciting light. Just 2 years later, E. Jette and W. West (1928) built the first photoelectric fluorometer. The other method (the direct flash method) of measuring lifetime of fluorescence was by using a flash of exciting light to excite the sample, followed by measurement of the decay of fluorescence in the dark with a weak measuring light. After photocells and photomultipliers had been invented, and improved, during the 1930–1950s, fluorometry of Chl *a* in vivo emerged as a major method in photosynthesis research (see chapters in Papageorgiou and Govindjee 2004 for the detailed use of fluorescence in photosynthetic systems).

During World War II, Coleman company had produced the first commercial fluorescence instrument. At that time, this instrument, as well as Beckman DU absorption spectrophotometer, were restricted to the use by the Military until the end of World War II. Moss and Loomis (1952) had constructed their own absorption spectrophotometer and were one of the first to measure absorption, transmission, and reflection in leaves of plants as well as in algae. For a review, see Carter and Knapp (2001). During 1955–1956, two companies had developed

spectrofluorometers; they were Aminco-Bowman (Silver Spring, MD, USA) and Farrand Optical Company (Walthalla, NY, USA) (see e.g., Bowman et al. 1955). In contrast to steady-state measurements, Steve Brody, in 1957, constructed the first device to measure fluorescence lifetimes in several photosynthetic samples at the University of Illinois at Urbana-Champaign, using direct flash method (Brody 1957; see Brody 2005, for a historical account), whereas in the same year, Dmitrievsky et al. (1957), in Russia, devised a phase method to measure the lifetime of fluorescence in the green alga *Chlorella*.

It is important to mention that Alexander Jablonski (1898–1980), known as “the father of fluorescence spectroscopy,” had introduced what is now known as the Jablonski Energy Diagram, a tool that is used to explain absorption and emission spectra as well as the paths taken by molecules that lead to PF, DF, and phosphorescence. Since this followed the earlier pioneering work of F. Perrin, the energy level diagram is called the Perrin–Jablonski diagram (see discussion in Valeur and Brochon 2001).

Strehler and Arnold (1951) invented the first apparatus to measure DF in photosynthetic systems (Fig. 4). Their experiments strongly suggested that this DLE by green plants is a reflection of certain early reactions in photosynthesis, which, by virtue of their reversibility, are capable of releasing a portion of their stored chemical energy through a “chemiluminescent” mechanism.

During the 1960s, more laboratory fluorometers were constructed and used in photosynthesis research, such as the instrument used by one of the authors (Govindjee), at the University of Illinois at Urbana-Champaign, during 1961–1963 (Fig. 5; for a diagram of this setup, see Shimony et al. 1967). Also see fluorescence instruments used by Warren Butler (Butler 1966).

During the 1970s and 1980s, many home-made instruments/systems were developed by several scientists. Some

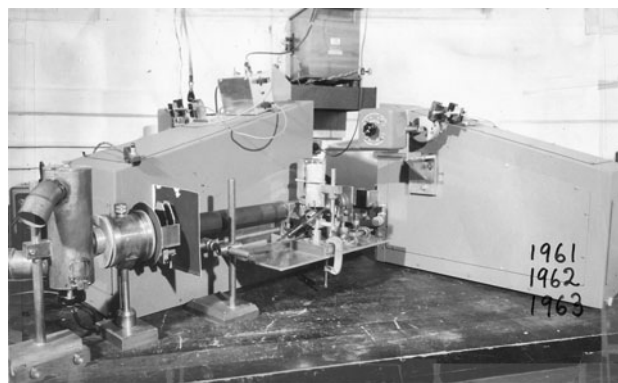


Fig. 5 A photograph of the spectrofluorometer that was built by Govindjee and Jobie D. Spencer in 1960; it was used from 1960 to 1963 for many discoveries including the discovery of the two light effect in chlorophyll fluorescence (Govindjee et al. 1960), and the discovery of a new emission band when photosynthesis was saturated (Krey and Govindjee 1963); it used 2 large Bausch and Lomb monochromators (see the sloping large units on the right and the left) for excitation and emission. When fluorescence transient was measured, white light filtered with a combination of Corning and Schott colored filters, or interference filters, was used from an optical system (see foreground). Readings were taken using a sensitive galvanometer (see box at the top), and later using a Brown chart recorder. Source A diagram of this instrument is in Shimony et al. (1967). Photo by Govindjee

examples follow. In addition to the use of just fluorometers, other instruments were developed by one of the authors (Reto Strasser) to measure other signals, originating from PSII, such as oxygen evolution and absorption changes of Hill reagents (artificial electron acceptors) (Strasser 1973a). With this setup, Strasser (1973b) studied the correlation of simultaneously measured variable fluorescence, DF, and oxygen evolution in leaves, just when the photosynthetic apparatus was initiated to begin functioning. Strasser and Sironval (1973) reported on the induction of PSII activity by measuring induction of variable part of the fluorescence emission in flashed bean leaves exposed to

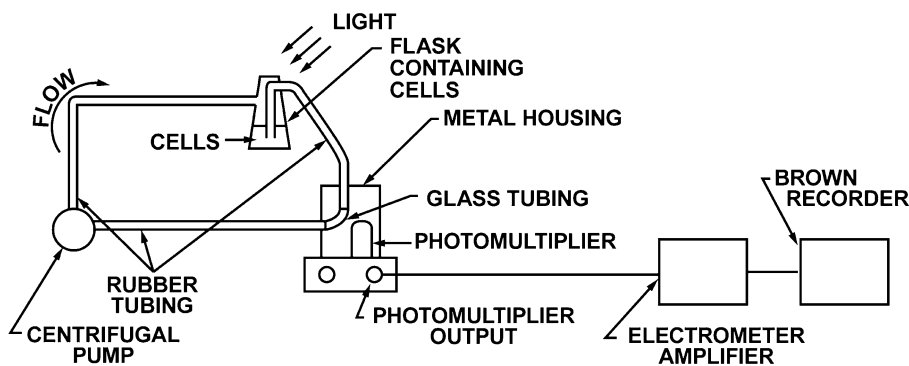


Fig. 4 A schematic diagram of an apparatus that was used to discover and measure DF (also called DLE) from suspensions of green algae (cells). In experiments on measuring DF, cells are illuminated at one time and at one place, and using a flow system,

they are brought, in darkness, before a photomultiplier. All parts of this 1951 instrument are clearly labeled in the diagram. Source Strehler and Arnold (1951)

weak green light. Soon thereafter, Strasser (1974) presented combined measurements on absorption, reflection, prompt and DF emission in flashed leaves. With improved methods and instrumentation, Strasser and Sironval (1974) provided quantitative parallel measurements on oxygen evolution burst and variable fluorescence in leaves. These multi-parameter measurements were made possible because of the use of the custom-made light guide fiber optics that had three arms. During 1980s, Walker and coworkers (Delieu and Walker 1983; Walker and Osmond 1986; Walker 1987) made parallel measurements on oxygen evolution and chlorophyll *a* fluorescence. Strasser (1986) observed an interesting behavior pattern, when he compared oxygen evolution with DF and PF: they observed both anti-parallel as well as parallel behavior between oxygen evolution and light emission. The instrument that Strasser had built, with Sironval in Belgium (see Fig. 6) was transported to the laboratory of Warren Butler in the USA. There, it was rebuilt and modified for excitation energy transfer measurements in flashed bean leaves, from the core antenna of PSII to PSI (Sato et al. 1976). For PSI activity, the kinetics of absorbance decrease at 705 nm (due to P700 photooxidation) was measured.



Fig. 6 The instrument room, in early 1974, in the laboratory of Warren L. Butler (1925–1984) at the University of California San Diego, California. It shows an instrument setup that was filled with oscilloscopes, chart recorders and photomultipliers. The instruments, shown here, were built and used by one of the authors (Reto J. Strasser; in the foreground). Specifically, the system had low voltage home-made oxygen monitors with amplifiers and external offset boxes to measure high amplification of O_2 in the zero to 8.0 ppm range, a 12-bit data acquisition with ms time resolution and two analog channels, analog and digital signal visualization, floppy disk drive, power supply, a tower with an oscilloscope for fast recording by single shot and polaroid photography, three pen recorders to monitor electric stabilities, fastest available X–Y recorder, available at that time, monochromators for excitation and emission spectra and photomultiplier tubes (EMI and Hamamatsu) with four high voltage supplies. From data, obtained with such instruments, Strasser and Butler evolved models of excitation energy distribution and redistribution in photosynthesis (see e.g., Butler and Strasser 1977). *Source* Photo was taken by an unnamed assistant in Butler's lab

For history, we mention some details of the instrument used by Sato et al. (1976), who measured Chl fluorescence at 685 nm with a home-made system: this instrument used Balzers 685 nm interference filter, two Toshiba V-R68 cut-off filters, and a S-20 EMI 9558 photomultiplier tube. Fluorescence was excited by a Ne–He laser system ($300 \mu\text{W}/\text{cm}^2$). We note that similar instruments had also been used for measurements of the photoreduction of NADP (Duysens and Ames. 1957; cf. Mi et al. 2000). This type of measurement enabled Sato et al. (1976) to study excitation energy transfer from PSII to PSI in chloroplasts (cf. Allakhverdiev et al. 2007b).

As an example of companies involved in producing equipment for fluorescence measurements, we mention Hansatech Instruments Company (Norfolk, England) with whom David Walker (see “Dedication” section) and two of us (HK and RJS) have been associated with; this company first became involved with the measurement of Chl *a* fluorescence in 1983, when they designed the fluorescence detector probe (FDP) associated with a control box. This was done in association with David Walker at the University of Sheffield, UK. In 1985, Hansatech also developed the transient recorder, TR1, which allowed fast fluorescence induction signals to be recorded, digitized, and replayed as an analog signal out to a chart recorder over an extended time base commensurate with the slow pen response speeds of chart recorders. Other instruments have also been developed by the same company, e.g., modulated fluorimeters (MFMS and 2-channel MFMS/2T) and the 4-channel MFMS/4T, recording two low light intensity modulated and the two high light intensity (due to actinic light excitation) fluorescence signals. (MFMS was the forerunner of instruments labeled FMS-1 and FMS-2.)

In 1990s, a small company “TEST” (Krasnoyarsk, in Russia) developed a fluorometer, labeled as FL3003; it provided excitation in the blue (400 nm), blue-green (515 nm), and green (540 nm) regions. This instrument was, thus, able to separate—green algae, blue-green algae (cyanobacteria), and diatoms in phytoplankton samples in natural ponds (Gaevsky et al. 1992). In 1992, another instrument, Photon 8 fluorometer, was also produced by the same company. This instrument allowed measurements of DF in various photosynthetic organisms: algal suspensions, isolated chloroplasts, plant leaves, pine needles, and lichens.

Earlier, several research groups had been building and using phosphoroscopes for Chl *a* DF measurements in the 1960s. One of the earliest instruments was by Walter Bertsch (Bertsch and Azzi 1965; Bertsch et al. 1967, 1969). Similarly, the use of devices for simultaneous measurement of PF and DF was underway in many laboratories at this time (late 1960s and beyond), notably in the laboratories of Louis N.M. Duysens (see e.g., Sonneveld et al. 1980a, b,

and references therein) and of Anthony R. Crofts (see e.g., Crofts et al. 1971, and references therein). We specifically mention a portable multi-flash kinetic fluorometer for measurement of donor and acceptor reactions of PSII in leaves of intact plants under field conditions by Kramer et al. (1990).

Venediktov et al. (1969) developed a phosphoroscope to measure DLE where there was a 3 ms time delay between illumination and the measurement of the emission from the sample (Matorin et al. 1976, 1978).

During 1976–1990, one of the authors, V. Goltsev, worked on another type of home-made phosphoroscope and instruments to measure both PF and DF (Fig. 7) (see later for a complete discussion of measurements from this instrument).

Several other instruments for measuring PF and DF have been built around the World, and have provided much data. In view of the fact that work published in non-English language is often not known internationally, we mention a home-made device, published in the Polish language, which was constructed and used by Antoni Murkowski and Aleksander Brzostowicz to measure DF kinetics from leaf samples in the 0.5–20 s range (Brzostowicz 2003; Brzostowicz et al. 2003; Murkowski 2002; Murkowski and Prokowski 2003).

Observations and measurements by portable instruments

Modulated fluorometers featuring mechanical choppers and lock-in amplifiers played an important role in basic photosynthesis research. In the 1970s, the technical progress in electro-photonics (availability of light-emitting diodes, LEDs, and fast photodiodes) led to an essential development in the area of Chl fluorescence instrumentation. The first portable fluorometer for field studies was that by Ulrich Schreiber et al. (1975). However, this instrument was limited to the recording of dark-light induction curves. Decisive stimulation leading to progress in instrumentation came from further physiological research of Briantais et al. (1979); Bradbury and Baker (1981); Krause et al. (1982); Horton (1983); and Walker et al. (1983). Chopper-modulated fluorometers were first applied for distinguishing different types of fluorescence quenching (Quick and Horton 1984; Dietz et al. 1985). Ögren and Baker (1985) and Schreiber et al. (1986) introduced the first portable fluorometer featuring modulated LEDs (Schreiber 2004). In 1987, Schreiber, for the first time, had a *Patent* that dealt with their “pulse-amplitude-modulated (PAM) fluorometer” (see data shown in Fig. 3), which had state of the art properties: distinguishing ambient and modulated light, modulated fluorescence excitation and high selectivity of the fluorescence amplifier for the modulated signal (Schreiber and Schliwa

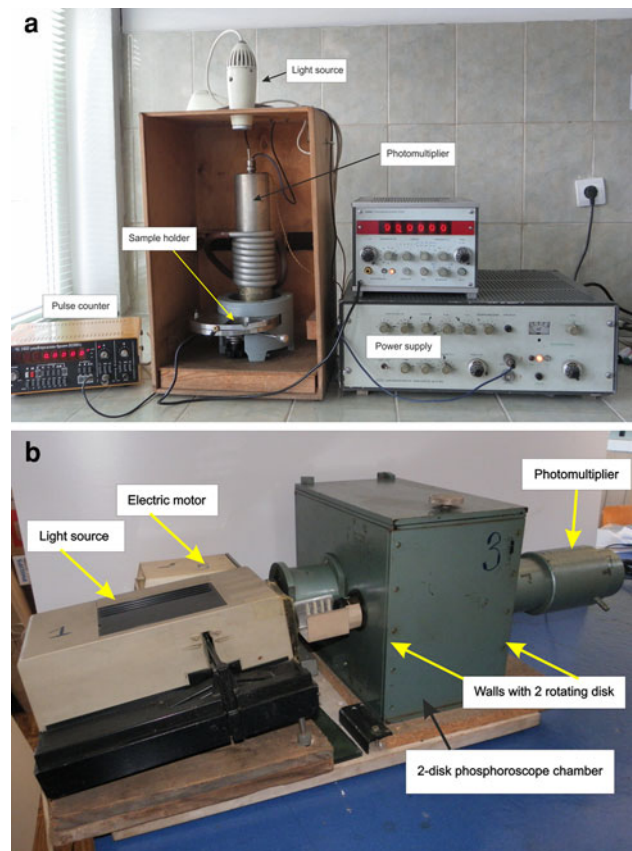


Fig. 7 DF instruments, used in the laboratory of one of the authors (VG). Since DF is discussed in greater details in this review, we have also provided details of the DF-measuring device. All the major parts of the instrument are labeled on the diagram for the instruments. *Top left* Experimental device for the recording of long-lived (0.5–100 s) DF after the actinic light was turned off. In this instrument, detached leaves or chloroplast suspensions (see sample holder) were illuminated for 20 s with light from a 40-W tungsten lamp (*top*) that passed through two colored glass filters (BG 18, Schott, Mainz, FRG and CS 5030, Corning, Rochester, NY, USA). The energy of the exciting light was 5 W m^{-2} . After illumination, the sample was mechanically moved toward the photomultiplier photocathode. Delayed fluorescence, which passed through a cut-off RG-630 filter (Schott), was measured using a photomultiplier type 79 (USSR; now Russia) with a spectral sensitivity curve of an S-20 type photocathode. DF signal was recorded both in photon counting (by a pulse counter; *left bottom*) and photocurrent modes (Yordanov et al. 1987). *Top right* Laboratory built device for millisecond DF recording. This fluorometer was designed as a two-disk-phosphoroscope of the Becquerel-type (produced at the Moscow University, USSR, now Russia). *Source* Photographs were taken by one of the authors (VG)

1987). The detailed technical specification of such a system is reviewed by Schreiber (2004); it is commonly used in saturating pulse (SP)-mode for the determination of fluorescence parameters associated with the slow F_i , including the measure of the recovery of the initial fluorescence yield after illumination pulses (see results obtained with PAM fluorometer by, e.g., Demmig-Adams et al. 1996). Prompt Chl *a* fluorescence measurements have been one of the most used as well as misused methods in the understanding of the

physiology of plants, algae and cyanobacteria. The relation between photosynthesis and fluorescence is quite complex and depends upon many factors. Logan et al. (2007) have discussed the common errors in the use of chlorophyll fluorescence on plants under field conditions. They advise the researchers to examine first the raw data traces before relying on automatically calculated parameters of the instruments used. More importantly, they emphasize that reliable interpretations are obtained when PF measurements are combined with measurements on photosynthesis, on chlorophyll concentration, and on concentrations and activities of photosystem I and II. Several chapters in Demmig-Adams et al. (2005) provide critical discussion of relevant problems that deal with the important question of photoprotection and photoinhibition when plants are exposed to excess light.

The next generation fluorometers provided the experimenter with very sensitive measurements of low Chl content samples, and fluorescence from single cells could be measured (Küpper et al. 2009). In this instrument, the problem of linearity over a large range of light intensities in the photodiodes (used as fluorescence detectors) and of maintaining a low noise level under extremely high light were solved by the use of pulse modulation, not only in the measuring light but also in the actinic light and in the saturation pulses (Schreiber 1998). Also, P700 absorbance was measured by the use of the same PAM technique (Klughammer and Schreiber 1998). Klughammer et al. (1990) developed a 16-channel LED-array spectrophotometer for measurement of time-resolved difference spectra in the 530–600 nm region to measure absorbance changes of cytochromes (Cyt *f*, Cyt *b563*, and Cyt *b559*). In the same year, Kolbowski et al. (1990) developed a computer-controlled pulse-modulated system for the analysis of photoacoustic signals. This technique was used by Reising and Schreiber (1992) to study pulse-modulated heat release, O₂ evolution, and CO₂ uptake associated with stromal alkalization (Schreiber 2004) and detection of thermal deactivation processes (Allakhverdiev et al. 1994).

An important fact is that the end of the *Cold War* between the USA and the Soviet Union (1991) was the reason to allow military secrets and advanced instruments, which had been kept only for the army, to be made available for civilian scientific research. That was the era of portability of the instruments in biology and physiology as well.

During the last 30 years, two leading worldwide companies, Hansatech Instruments Ltd. (UK) and Heinz Walz GmbH (Germany), have developed many lab and portable fluorometers to measure Chl *a* fluorescence from intact leaves and from algal suspensions in liquid media (see <http://www.walz.com/>; for history: <http://www.walz.com/company/history.html>). This was a result of long-term cooperation and scientific support from the late David Walker (1928–2012; see Edwards and Heber 2012) and Reto Strasser to the Hansatech Company, and Ulrich

Schreiber to the Walz Company. Both companies have delivered a very professional set of instruments. This was the main reason behind the very fast advances in research in using Chl *a* fluorescence during the last three decades. Currently, another emerging company, which has the conceptual and technical support of Ladislav Nedbal, is photon systems instrumentation (for information, see <http://www.psi.cz/about-psi/our-company>.) Furthermore, there are multi-wavelength kinetic fluorometers (MWKF), based on photodiode array detectors, which provide three-dimensional (3D) fluorescence induction kinetics (F vs λ vs t). See Kaňa et al. (2009, 2012) for further details and application of this instrument.

Kolber et al. (1998), on the other hand, devised a fast repetition rate (FRR) method to measure variable fluorescence and have applied it successfully to many photosynthetic systems. Ananyev and Dismukes (2005) applied the FRR technique to even monitor the speed with which PSII can split water (cf. Shinkarev et al. 1997 who had obtained kinetics of these steps through chlorophyll *a* fluorescence measurements after single saturating light flashes). Further, Kolber et al. (2005) have used a laser-induced fluorescence transient (LIFT) to remotely monitor terrestrial vegetation. Using LIFT and remote sensing, Ananyev et al. (2005) were able to measure heterogeneity in photosynthetic efficiency, electron transport, and dissipation of excess light in *Populus deltoides* stands under ambient and elevated CO₂ concentrations. Special instruments, with multiple functions, are being used in studies on photosynthesis. The use of these instruments in photosynthesis research is expected to bring answers to many important open questions related to specific processes that influence the quantum yield of Chl *a* fluorescence, and, thus, the quantum yield of photosynthesis. We note that some of these devices, manufactured by Walz Company, such as the Dual-PAM-100 (measuring signals from P515 and P700), and KLAS-100 (measuring signals from Cyt *f*, C550, Cyt *b559*) are highly useful. The new multi-color-PAM (Schreiber et al. 2012) is a welcome addition: it is a new tool with special applications in the study of the OJIP rise kinetics. Hansatech Company produces also advanced instruments of this type, such as the multi-function plant efficiency analyser (M-PEA) that combines for the first time a high quality fast PF kinetic and P700-modulated reflectance (MR) signals with DF measurements and leaf absorptivity, which will be presented in more details below.

The M-PEA instrument, example of a multi-signal instrument

A recently developed instrument is M-PEA, from Hansatech Instruments Ltd, a company that David Walker had collaborated with on many other instruments, as already

mentioned (http://www.hansatech-instruments.com/electrode_chambers.htm). The M-PEA (Fig. 8; also see Strasser et al. 2010) combines, for the first time measurements of prompt and delayed chlorophyll fluorescence signals, with transmission (reflectance) changes at $\lambda = 820$ nm (for P700). It uses a relatively small optical sensor unit (working head) and a sample holder (“clip”) that allow(s) the investigation of plants. In this instrument, all of the light sources and detectors are put together in the sensor unit itself, and are covered by a quartz window; this protects the instrument from dust, dirt, and moisture. A bright light-emitting diode provides high-intensity red actinic light; the instrument includes a far-red light source for preferentially exciting PSI. Further, M-PEA includes also a high sensitivity DF detector, as well as a detector to measure leaf absorptivity (For all information on Hasatech products, go to <http://www.hansatech-instruments.com/>).

For completeness, some details follow: (1) *PF measurements in M-PEA*. The PF signal detection system for the M-PEA uses a high-intensity red LED up to $5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the sample surface for effective light saturation of the sample. The emitted fluorescence signal is captured by a low noise, fast response PIN photodiode with the detected signal being processed

by a high performance 16 bit A/D converter for optimum resolution; (2) *Modulated P700 absorbance/reflectance measurements in M-PEA*. The M-PEA uses an optically filtered, modulated 820 nm LED for high quality P700 reflectance measurements. P700 activity is recorded using an optimized low noise, fast response PIN photodiode; and a 16-bit A/D converter providing an excellent signal-to-noise ratio even in the ms and sub-ms region. Measurements of PF and P700 are plotted on the same axes in the M-PEA⁺ software (see further details below); (3) *Leaf absorptivity measurements in M-PEA*. The leaf absorptivity element is effectively a measurement of the “greenness” of the leaf, giving a relative indication of Chl content. M-PEA uses the actinic, the actinic far red, and the modulated 820 nm light source to give an absorptivity measurement. All fluorescence intensity measurements depend on the actual light energy flux absorbed, which may change during experiments lasting for several seconds to minutes (e.g., due to light scattering changes after possible turgor changes and due to the movement and alignment of the chloroplasts in the cells). The M-PEA is an instrument where the actinic light energy absorption is measured before, after or during illumination period with continuous PF, DF and MR (Modulated light reflectance at 820 nm) data acquisition. This allows one to distinguish changes in absorption properties from fluorescence quenching properties; (4) *DF measurements in M-PEA*. As mentioned earlier, DF is the light that is emitted from green plants, algae, and photosynthesising bacteria for a short time after they have been exposed to light, but after the PF emission has decayed. DF occurs, as mentioned earlier, in the red-infrared region of the spectrum (the same as prompt chlorophyll fluorescence). However, again, as mentioned earlier, the intensity of the DF emission is lower than that of PF by at least two orders of magnitude, therefore, requiring extremely high sensitivity apparatus to measure the signal. M-PEA features an optically filtered, highly sensitive wideband avalanche photodiode for the measurement of the DF signal. The system can be configured to make DF measurements at regular intervals during a PF recording or as a terminal measurement at the end of a PF induction.

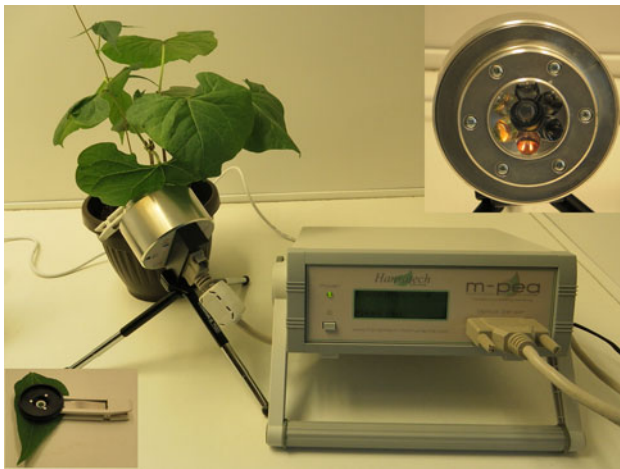


Fig. 8 M-PEA (of Hansatech company, UK). It allows one to measure several signals: PF (Chl *a*), DF (Chl *a*), P700 absorbance change, and relative chlorophyll content. The device electronically separates the signals of prompt and DF using fast switching light-emitting diodes as the light sources, and sensitive photodiodes as the light sensors. The light emitters and sensors are placed in an optical sensor unit (shown as *inset* on the *top right corner*). The optical sensor unit is fixed on a tripod and has 3D mobility, making it convenient to use different plant samples. On the sample holder (leaf clip, see *inset* on *bottom left corner*), leaf is fixed to the optical sensor unit; further, the sample holder protects the leaf and the sensors from extraneous light. The figure also shows a 30-day-old decapitated bean plant (ready to be used for an experiment; see Yordanov et al. 2008). Photograph by one of the authors (VG)

Further details of simultaneous PF and DF measurement in photosynthetic samples and reconstruction of their induction curves are given in the *Appendix* by one of us (VG) for the benefit of advanced students.

With this instrument (the M-PEA), one measures simultaneously the PF and the MR at 820 nm, related to P700 changes (Schansker et al. 2003); the light phase of a PF transient can be interrupted by short dark intervals, ranging from μs to ms, during which the DF kinetics are recorded with the same data acquisition system as PF and MR. The recombination reactions that provoke the DF

signals depend on the redox state of the PSII primary electron quinone acceptor (Q_A), which is reflected in the relative variable PF $V_t = (F_t - F_o)/(F_m - F_o)$. The redox state of the primary quinone electron acceptor of PSII Q_A depends on the redox states of the electron transport chain carriers, which, in turn, depends on the redox state of PSI RC (P700) that determines the 820 nm light reflection (MR) kinetics. Therefore, the simultaneous in vivo measurements of PF, DF, and MR allow collection and correlation of complementary information for all three domains of the photosynthetic electron transport—PSII electron donor side, electron transport between PSII and PSI, and PSI electron acceptor side (Bukhov and Carpentier 2003; Rajagopal et al. 2003; Strasser et al. 2004, 2010; Tsimilli-Michael and Strasser 2008). In most measurements on leaves, excitation, emission, and modulated measuring beams are directed toward, or away from the leaves, on one side (usually the upper leaf side) only. This way, by measuring leaf disks, the lower side of the leaf is free and available for simultaneous measurement of oxygen gas exchange in the second to minutes time range. This has indeed been done with an adapted Hansatech Clark-type oxygen electrode. For a technical arrangement, see Strasser (1974), Strasser and Sironval (1974), and for recent data, see Gururani et al. (2012).

Comparison of PF and DF measured simultaneously

As we already know, light quanta, both in prompt and delayed Chl fluorescence, are emitted from the same population of PSII antennae Chl molecules. The opportunity to experimentally record both types of light emission by the same sample at almost the same time allows their comparison and an understanding of the similarity and the differences between the two signals, and may also provide additional information about the state of the photosynthetic machinery. The DF induction curve is often compared to the PF transient presented on the same time scale to obtain an insight into the nature of the maxima in the two processes (Govindjee and Papageorgiou 1971; Krause and Weis 1991; Malkin et al. 1994; Goltsev et al. 2009; Strasser et al. 2010). The problem of comparison of DF with PF transient is that the ms DF usually is a complex mix of fast and slow kinetic components, which behaves in a different way during the induction period (Mar et al. 1975). The ms DF, with a lifetime of 2–3 ms, does not correlate with the changes in PF, while for the longer DF components, such a correlation has been observed (Clayton 1969; Malkin and Barber 1978).

A good approach for visualization of PF and DF correlation is to present them as what may be called a *phase diagram* (Malkin et al. 1994). In a 2D graph, DF within different dark decay intervals is plotted as a function of

relative variable fluorescence V_t (see further discussion in the Appendix by one of us, VG). Every DF point is an averaged value of DF signal collected from one of the three dark time windows: 20–90 μ s (Fig. 9, left, a), 100–900 μ s (Fig. 9, middle, b) and 1–2.3 ms (Fig. 9, right, c). DF points are plotted against corresponding values of PF recorded just before dark interval used for DF measurement. Each of the three “phase diagrams” can be divided into two parts—a non-linear part reflecting points belonging to the OJIP part of PF transient and I_1 – D_2 part of DF induction, and a linear part where points of slow phases of PF and DF lie. (For the I_1 – D_2 part of DF, see a paper by Ganena et al. 1988.) In the linear part of the curves, PF and DF change in the same way: thus, we may assume that the main cause of these changes is related to fluorescence quantum yield (Lavorel 1975; Goltsev et al. 2003). In the fast phase, PF and DF deviate from linearity because the photosynthetic reactions determining PF and DF changes affect them in different ways. Four parts of the phase diagram, that are characterized with specific type of correlation between DF and the variable PF, V_t , could be separated (see Fig. 9): (1) PF and DF increase simultaneously at the beginning of the induction curve (DF phase O– I_1 , time interval $t = 0.3$ –11 ms); (2) DF decreases as the PF increases (DF phases I_1 – I_2 – I_3 , time interval $t = 11$ –300 ms); (3) DF increases as PF decreases (DF phases I_3 – I_4 , time interval $t = 0.3$ –5 s); and (4) PF and DF are linearly correlated during simultaneous decrease of both signals within the time interval of 5–300 s (DF phases I_4 –S). These are complex relationships and further research is needed to exploit this method and to understand these observations.

The first type of correlation is mainly expressed in the phase diagram for the ms component of DF. The well-pronounced lag phase before the steep DF increase may imply that the formation of DF precursors $S_3Z^+P680Q_A^-$ requires absorption of at least three light quanta in each PSII. The second type of correlation during DF change from the I_1 to I_3 reflects the closure of PSII RCs, and the formation of states $S_2ZP680Q_A^-Q_B^{2-}$ that can produce slow DF component, but cannot produce fast μ s decaying component. The third type of correlation occurs during the I_3 to I_4 DF induction phase, and, perhaps, this is the result of photoinduced thylakoid membrane energization that affects PF and DF in different ways.

Concluding remarks on comparison of PF and DF

Once light energy is absorbed by Chl *a* molecules of the antenna complexes, it undergoes a series of successive transformations before it is converted into free energy of chemical bonds in carbohydrates. Most light reactions of the photosynthetic process are principally reversible and the energy can be returned at any stage to its initial form

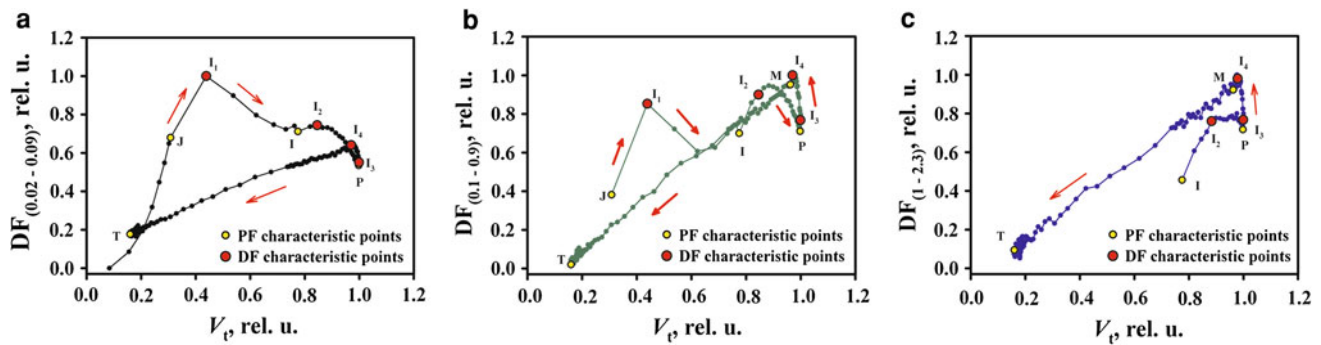
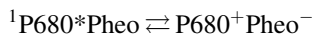


Fig. 9 Diagrams showing correlation between DF and relative variable PF, V_e . **a** (Left) Microsecond (20–90 μ s) DF, **b** (middle) sub-millisecond (100–900 μ s) DF; and **c** (right) millisecond (1–2.3 ms) DF. Characteristic points of DF induction (labeled as I_1

... I_4) are marked with *solid (red) circles* and of PF transient points (labeled as $J, I, P, (S), M,$ and T) are marked with *solid yellow circles* (see text for details, including meaning of the *symbols*). This figure was drawn by one of the authors (VG), using his own original data

and emitted as light quanta of PF and DF. The re-emitted quanta of PF and DF radiation contain very important information about the forward and backward reactions that lead to the formation of the excited state of the antenna Chls.

In summary, before PF emission, a series of primarily photophysical processes occur: (1) absorption of light energy and excitation of Chl molecule; (2) internal conversion of energy in the excited molecule; (3) migration of excitation energy within and between neighboring Chl protein complexes; (4) establishment of excitation energy equilibrium between antenna Chls and the Chls of the reaction center (Dau and Sauer 1996; Grabolle and Dau 2005); and (5) reversibility of the reaction generating the radical pair $P680^+Pheo^-$ (Schatz et al. 1988):



PF provides information about the structure and energy migration processes within photosynthetic antenna (Clegg et al. 2010). But the redox equilibrium in the PSII reaction center is strongly determined by subsequent redox reactions in the electron transport chain, and that allows using fluorescence to “see” different parts of the electron transport chain—from PSII acceptors, plastoquinone pool, and even the electron transfer from the PQ pool to the PSI terminal acceptors (see Strasser et al. 2004, 2010; Tsimilli-Michael and Strasser 2008; cf. Schreiber and Neubauer 1987; Papageorgiou and Govindjee 2011; Stirbet and Govindjee 2011).

Regardless of the fact that the same population of antenna chlorophylls of PSII emits the PF and DF, they carry different, complementary information about the quantitative characteristics of the photosynthetic process. DF quanta are emitted after a series of photophysical and photochemical reactions followed by chemical redox reaction both on the donor and the acceptor side of PSII. Therefore, DF emission carries additional information not

only about the concentration of the PSII redox states—the DF precursors but also about the rates of electron transport reactions in which they participate (Lavorel 1975; Goltsev et al. 2005, 2009). Thus, DF is the source of information about free-energy differences between the excited-antenna state and the radical pair state (Grabolle and Dau 2005); differences in energies of several PSII redox states can be calculated reasonably precisely. Based on the measured DF decays, the redox-potentials of the electron carriers on the PSII donor side have been evaluated (Grabolle and Dau 2005; Zaharieva et al. 2011). The DF emission of PSII is a useful tool to quantitatively study light-induced electron transfer and related processes (e.g., proton movement; Dau and Zaharieva 2009, Zaharieva et al. 2011), which are associated with a free-energy drop (Buchta et al. 2007).

The measurement of PF and DF emitted simultaneously from a sample (whether in vitro, in vivo or in situ) has opened a new perspective for using these emissions as tools for photosynthesis research in vivo. Information from both the signals could be summarized, compared, and inter-checked to provide a better view of the mechanisms of both types of light emission (PF and DF) and to obtain further details of the photosynthetic machinery, its structure and function.

Concluding remarks

One of the greatest uses of PF has been in understanding excitation energy transfer from various photosynthetic pigments to Chl *a* (using the well-known “sensitized fluorescence” method). This method was first used by Cario and Franck (1922). When a mixture of mercury (Hg) and thallium (Tl) vapor was excited with the light absorbed by Hg (254 nm), they observed emission spectra of both Hg and Tl; since Tl was not excited, it could give light emission (at 535 nm) only because of excitation energy

transfer from Hg. This, then, was the first example of sensitized fluorescence (see Cario and Franck 1922; Loria 1925 for confirmation and extension of the concept). The method of sensitized fluorescence was elegantly used in the 1952 doctoral thesis of Louis N.M. Duysens at the State University, Utrecht, The Netherlands (Duysens 1952). (A powerpoint presentation on Forster energy transfer (FRET) is available from a site in the Czech Republic, by searching for “UFCH_fluor07.pps”.) Govindjee et al. (1960) confirmed, through the observation of quenching of PSII fluorescence by PSI light, the existence of two light reaction and two pigment systems, as implied in the discovery of enhancement effect on oxygen evolution (Emerson et al. 1957). Duysens and Sweers (1963) provided the key hypothesis that Chl *a* fluorescence intensity is inversely related to the concentration of Q_A , the first plastoquinone electron acceptor of PSII (see Strasser 1978; Strasser et al. 2010; and see Stirbet and Govindjee 2012 for a full discussion, including its shortcomings). Fluorescence is a sensitive and non-invasive indicator of photosynthesis, but only parallel and simultaneous measurements on fluorescence, oxygen evolution, CO₂ fixation, and partial reactions of the entire photosynthetic chain can provide the full breadth of understanding of the phenomenon under investigation. Instruments exist for measuring photosynthesis and chlorophyll fluorescence in the same system (see e.g., CIRAS-2 at http://www.ppsystems.com/ciras2_portable_photosynthesis_system.htm and LI-COR's LI-6400XT at <http://www.licor.com/env/applications/fluorescence.html>). However, time has come for recommending to the major manufacturing companies to accept the challenge of producing inexpensive instruments for measuring simultaneously, and in parallel, fluorescence, whole chain electron flow, PSII and PSI activities, O₂ evolution and CO₂ uptake on algae, cyanobacteria and plant farms since the future goal of obtaining biomass, biofuel and bioenergy depends on the efficiency of photosynthesis at all levels.

We have traveled a winding path from the earliest observations of Sir G. G. Stokes (1819–1903) to our current status of using light emission as a tool to understand the complex photosynthesis machinery. We end this historical and educational review by expressing our appreciation to David Walker (1928–2012) who was a master of communicating basic concepts of these processes to children as well as the elders.

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Appendix 1 (by V. Goltsev)

DF measurement

In view of the not-so-common use of DF, we describe here in some details this method and analysis of data. Two experimental approaches are used for the measurement and the analysis of the DF signal: (a) monitoring of relaxation of DF intensity in the dark (the so-called “dark DF decay”); and (b) recording of “DF induction curve” (IC) during the transition of dark-adapted samples to light-adapted state. In the first approach, the samples are pre-illuminated by short (single turnover) light pulse (flash) or by continuous light to form redox states of PSII that lead to emission of DF, for example $S_3Z^+P680Q_A^-Q_B$. Here, S_3 is one of the oxidized *S*-states of the oxygen evolving complex of PSII (Joliot and Kok 1975). After turning off the actinic light, the rate of DF quanta emission and the kinetics of DF decay are analyzed. Such an approach is usually applied for measuring the DF kinetic components decaying in ns (Christen et al. 2000), in μ s (Grabolle and Dau 2005; Buchta et al. 2007), in ms (Goltsev et al. 1980) or in s (Rutherford et al. 1984; Rutherford and Inoue 1984). To evaluate long-lived light emission (>seconds or minutes), the samples are excited by continuous light (Hideg et al. 1991; Katsumata et al. 2008; Berden-Zrimec et al. 2008).

When a DF emission is monitored during illumination of a dark-adapted sample by continuous light, the DF induction curve is measured, as is done for recording the OJIP transients of PF. Both the measurements reflect changes in the photosynthesis machinery during dark-to-light adaptation. The same population of Chl-proteins of the PSII antennae complexes that emit PF emits DF quanta. The main difference is that the quantum yield of PF is 3-10

orders of magnitude higher than that of DF, and the DF quanta cannot be distinguished from those of PF during illumination. One effective experimental approach that allows one to distinguish between the two types of light emission is the separation of the two processes as follows: PF is recorded simultaneously with illumination and DF—after turning off the actinic light. For measurement of DF induction, it is necessary to use alternate light/dark cycles. During the light period, PF can be measured, a short time interval after the light is turned off (to avoid measuring PF), DF dark decay is measured (see Fig. 10).

During each dark interval, the DF signal shows a polyphasic decrease. In most analog phosphoroscope-based DF-measuring devices, the quanta emitted during each dark interval are collected, integrated and presented as a value proportional to DF intensity at definite times. The time course of the measured signal at intermittent illumination of dark-adapted samples is presented as a DF induction curve. The digitalization of the measuring signal (Gaevsky and Morgun 1993; Zaharieva and Goltsev 2003) and the use of fast analog–digital converter devices ($<50 \mu\text{s}$) allows analysis of the kinetics of DF relaxation at each dark

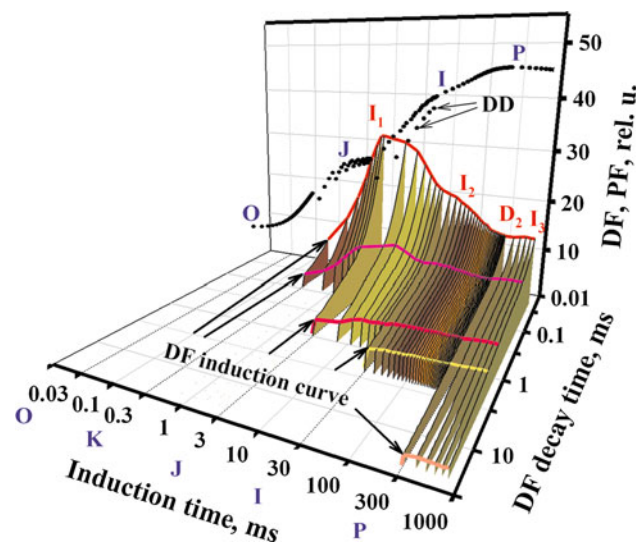


Fig. 10 Reconstruction of kinetics of prompt (chlorophyll) fluorescence (PF) and delayed (chlorophyll) fluorescence (DF) signals measured simultaneously by a M-PEA instrument (Hansatech, UK; see Fig. 8) device during dark-to-light adaptation in bean leaves. The data acquisition for the two signals, PF in the light and DF in the dark, was every 0.01 ms in the 0–0.3 ms range, every 0.1 ms in the 0.3–3 ms range, every 1 ms in the 3–30 ms, and up to 30 s; after this time, data was acquired every 10 s. The black dots show values of PF signal, at specific points, during the O–J–I–P transition—i.e., on the PF induction curve (see the *top vertical panel*). Points marked as DD (“dark drops”) show the first PF values recorded after short dark periods during which DF was measured. Sheets perpendicular to the back plane show DF dark decays at different times of the O–J–I–P transition. *Arrows* show DF induction curves, recorded at different decay times. This figure was drawn by two of the coauthors (VG and RS) from their own original data

interval during induction (Fig. 11). To construct DF induction curves, a distinct dark time period is chosen within which the values of DF intensities are averaged and used as a single point of DF induction curve. Selecting points from different decay intervals, one can construct induction curves that show DF kinetic components with different lifetimes. In Fig. 11, five DF induction curves are shown that used the following time points in DF decay curves, i.e., after 20 μs , 90 μs , 0.9, 2.3 and 23 ms of the start of dark interval. Thus, the time course of different components (measured at different delay intervals) of the DF decay can be monitored during the dark-to-light transitions.

Origin of DF induction phases

The DF induction curve reflects processes that occur in the photosynthetic machinery of plants during illumination after a period of dark adaptation. Usually, induction maxima are well pronounced after 5 to 15 min of dark adaptation. A stationary level of DF is reached after the 2–3 min of actinic light (Veselovskii and Veselova 1990; Radenovic et al. 1994).

The DF induction curve is extremely complex: it is multiphasic. Even 61 years after its discovery (Strehler and Arnold 1951), the reasons for the changes in the intensity of delayed light quanta emission during the induction transients, are not clear. DF intensity passes through several maxima and minima before reaching a stationary level. The main factors affecting the DF induction shape are: (1) The photosynthetic sample: plant species; (2) structural status of the sample (whole plant, isolated chloroplast suspension, membrane particles); (3) physiological state of the sample (chemical and physical treatments); (4) measurement details: e.g., dark adaptation duration; actinic light intensity; recording period (duration of time interval when DF is measured; dark interval before DF recording). Thus, measuring conditions determine which kinetic components of DF are being measured in an experiment (Zaharieva and Goltsev 2003).

There is no consensus nomenclature of the maxima that are observed in the DF induction curve, and there is no consensus about the number and interpretation of these maxima. We use here the nomenclature proposed by V. Goltsev and coworkers (Goltsev and Yordanov 1997; Goltsev et al. 1998, 2005, 2009; Zaharieva and Goltsev 2003) where the maxima (denoted by I) and minima (denoted by D) are numbered in a sequence according to their position in the DF induction curve (I₁, D₁, I₂, D₂).

The DF induction curve is easily divided into two main phases, a fast phase and a slow phase (Itoh et al. 1971; Itoh and Murata 1973; Malkin and Barber 1978) (Fig. 11). The fast phase that lasts for about 300 ms coincides with the

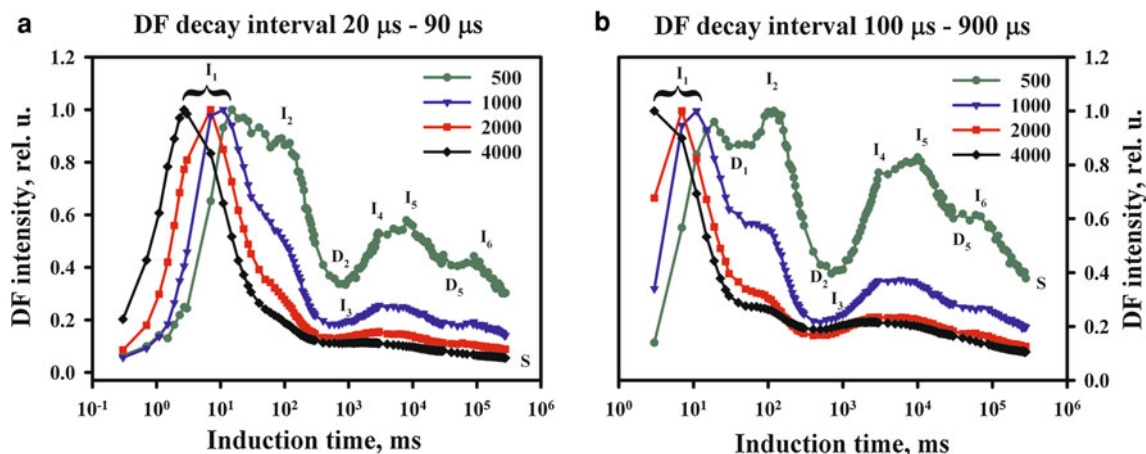


Fig. 11 Induction curves of delayed fluorescence (DF) recorded in 20–90 μs (left panel) and in 100–900 μs dark decay window (right panel) as a function of actinic light intensity. Primary leaves of decapitated bean plants were dark adapted for 1 h and then illuminated by red (625 nm) actinic light of different intensities from

500 to 4,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. DF intensities are normalized to maximal values for each curve. The “I’s” (inflection peaks) refer to the induction maxima, and “D’s” (dips) to the minima. This figure was drawn by one of the authors (VG), using his own original data

OJIP transient of PF, and then there is the slow phase that occurs in the minute range, reaching a stationary level at the end. Using a mechanical phosphoroscope with fast signal digitalization ($\sim 50 \mu\text{s}$) and electromechanical light “cutter” (opening time $< 1 \text{ ms}$), it is possible to resolve details in the structure of the fast phase. Thus, when DF is measured starting with 5.5 ms of illumination (the working cycle being 11–5.5 ms light and 5.5 ms dark and induction, see Goltsev et al. 2003), two maxima I_1 and I_2 (sometimes with a minimum D_1 in between) are observed in the fast phase; after this DF drops to a minimum labeled as D_2 (Goltsev and Yordanov 1997; Goltsev et al. 1998, 2003). After a small step, labeled as I_3 , the slow phase begins. During this phase, DF rises to a maximum I_4 and then, through several transient maxima (I_5 and I_6), DF intensity decreases to a stationary level S (Itoh and Murata 1973; Goltsev et al. 2003).

For DF that decays in 100- μs time interval, the time position of the first induction maximum I_1 as well as a ratio of I_1/I_2 are highly dependent on light intensity. At 4,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ the I_1 maximum appears at about 3 ms of illumination and it is shifted up to 15 ms at lower light intensity (500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A similar effect is observed in the induction curve of sub-millisecond DF component (Fig. 11, right panel).

When DF is compared with PF transient and with the kinetics of the signal of “reflection” of modulated light at 820 nm (called MR820—this photoinduced signal is caused by the appearance and disappearance of the oxidized form of P700 and of plastocyanin, see Schansker et al. 2003), the maximum I_1 coincides with the PF increase from the J-level (F_j) to the I level (F_I) and with decrease of MR820 reflecting P700 and plastocyanin oxidation (Schansker et al. 2003).

The growth of DF intensity up to I_1 probably reflects the accumulation of $S_3ZP680^+Q_A^-$ and $S_3Z^+P680Q_A^-$ states that have a relatively high yield of DF emission. The kinetics of DF decreases after the maximum I_1 to the minimum D_2 is similar to that of the PF rise from J to I and P phases, and it, possibly, is caused by the formation of “closed” PSII states $S_iZP680Q_A^-Q_B^{2-}$ that are not able to do charge recombination in sub-ms and ms time interval and, thus, DF formation. Another process that probably has a part in the kinetics of the fast phase of the DF induction is photooxidation of P700 and of plastocyanin (PC) as a result of the activity of PSI due to the lack of electrons in the plastoquinone pool (Schansker et al. 2003). The accumulation of positive charges in the inner part of thylakoid membrane in the form of $P700^+$ and PC^+ may lead to the formation of a transmembrane potential (Satoh and Katoh 1983). Thus, the appearance of I_1 , like the transition from F_o to F_j and F_I , can be related to two phenomena: (1) *photochemical*—accumulation of certain light-emitting states of the PSII RC, and (2) *non-photochemical*—increase in the DF due to the electrical gradient formed by PSI when P700 is oxidized (Pospisil and Dau 2002; Vredenberg et al. 2006).

The I_2 maximum (usually, at high light intensities; visible only as a shoulder) is probably related to the prolonged reopening of PSII RCs by the accelerated electron transfer from the reduced Q_B when the PQ pool is actively reoxidized by PSI before the full reduction of the PQ pool (I_2 – D_2 transition in the DF induction curve that coincides with the I–P phase in PF transient and with the slow increase phase in the MR820 (Modulated light Reflectance at 820 nm) signal. The relative size of this maximum depends on the ratio between the flow of excitation trapping in the RCs of PSII and of the intersystem electron transfer. The share of I_2 increases under

several conditions: at lower actinic light, with the decrease in the size of the PSII antenna; and with increase in temperatures (Zaharieva et al. 2001).

After about 300–500 ms of illumination, the plastoquinone pool is reduced and most of the Q_A is in its reduced state, Q_A^- . Chl fluorescence is maximal (P step) and MR 820 signal reaches its maximal level. At this moment of induction (phase D_2), DF is emitted from RCs in “closed” state $Z^+P680Q_A^-Q_B^{2-}$ (Gaevsky and Morgun 1993; Zaharieva and Goltsev 2003; Goltsev et al. 2005). This is “deactivation” type of light emission (see the main text) and is a result of charge recombination in $Z^+P680Q_A^-Q_B^{2-}$ or $S_1ZP680Q_A^-Q_B^{2-}$ PSII states. During this induction phase, the amplitude of the sub-ms DF components decreases, and the lifetime of the ms component increases (Zaharieva and Goltsev 2003). In the presence of an artificial electron acceptor (potassium ferricyanide) and uncouplers of phosphorylation, this increase in the lifetime of DF is insignificant and no I_2 – D_2 is observed. This indicates that the I_1 – I_2 – D_2 phase correlates with the processes of reduction of the PQ pool, and the JIP phase of PF transient (Schansker et al. 2003).

The peak I_3 was first discovered with a phosphoroscope-based DF instrument with low actinic light ($\sim 1,200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Goltsev et al. 2003) but it is not visible if DF is recorded at high actinic light ($4,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$); it is visible as a small shoulder after the D_2 phase in the DF induction curve with exposure to $1,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity. The source of DF emission of this phase is weakly luminescent “closed” PSII states.

The increase of DF to the next maximum, labeled as I_4 , is well pronounced at relatively low excitation light. The DF growth during the D_2 – I_4 phase coincides with a slight decrease in the PF intensity and reduction of MR signals caused by oxidation of P700 (Goltsev et al. 2005). The accumulation of $P700^+$ suggests that at this time the light-induced activation of the ferredoxin: NADP⁺-oxidoreductase takes place (Harbinson and Hedley 1993; Schansker et al. 2003), i.e., the linear electron transport is activated, and the transmembrane proton gradient starts to accumulate. The increase of the DF intensity in the slow phase (toward the I_4 maximum) is associated with the formation of a proton gradient (Wraight and Crofts 1971; Evans and Crofts 1973) that increases the rate constant of radiative recombination in the PSII RCs.

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