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In Vivo Measurements of Light Emission in Plants[a]

Hazem M. Kalaji[1], Vasilij Goltsev[2], Marián Brestič[3,*], Karolina Bosa[4], Suleyman I. Allakhverdiev[5], Reto J. Strasser[6], and Govindjee[7,*]

[1]Department of Plant Physiology, Faculty of Agriculture and Biology, Warsaw University of Life Sciences, SGGW, Nowoursynowska 159, 02-776, Warsaw, Poland;
E-mail: hazem@kalaji.pl

[2]Department of Biophysics and Radiobiology, Faculty of Biology, St. Kliment Ohridski University of Sofia, 8 Dr. Tzankov Blvd., 1164 Sofia, Bulgaria;
E-mail: goltsev@gmail.com

[3]Department of Plant Physiology, Slovak Agricultural University, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic;
E-mail: marian.brestic@uniag.sk

[4]Department of Pomology, Faculty of Horticulture and Landscape Architecture, Warsaw University of Life Sciences, SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland;
E-mail: karolinabosa@gmail.com

[5]Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, Moscow 127276, Russia;
Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia;
E-mail: suleyman.allakhverdiev@gmail.com

[6]Bioenergetics Laboratory, University of Geneva, CH-1254 Jussy/Geneva, Switzerland;
Weed Research Laboratory, Nanjing Agricultural University, Nanjing, China;
Research Unit Environmental Science and Management, North-West University Potchefstroom, South Africa;
E-mail: Reto.Strasser@unige.ch

[7]Department of Biochemistry, Department of Plant Biology, and Center of Biophysics & Computational Biology, University of Illinois at Urbana-Champaign, 265 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801-3707, USA;
E-mail: gov@illinois.edu

[*]Corresponding authors: Telephone: 1-217-337-0627; fax: 1-217-244-7246; E-mail: gov@illinois.edu; and Marián Brestič, Telephone: +421 905 416 163; E-mail: marian.brestic@uniag.sk, marian.brestic@gmail.com

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This chapter is dedicated to a pioneer in the field of photosynthesis and chlorophyll fluorescence: David Walker. For a Tribute to Walker, see G. E. Edwards and U. Heber (2012) David Alan Walker (1928–2012) Photosynth. Res. 112: 91–102. For an obituary and photographs, see R. C. Leegood and C. H. Foyer (2012) David Alan Walker. Am. Soc. News Letter 39 (5): 41–42. Also see a web site of Hansatech Instruments concerning oxygen electrodes named after him: (http://www.hansatech instruments.com/electrode_chambers.htm). Walker's contributions were substantial, 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). See David Walker's photograph on one of the following pages.

Abstract. There are several types of light emission in plants: prompt fluorescence, delayed fluorescence, thermoluminescence, and phosphorescence. This chapter focuses on two of them: prompt and delayed fluorescence. Chlorophyll *a* fluorescence measurements have been used for more than 80 years to study photosynthesis; since 1961, it has been used, particularly, for the analysis of Photosystem II (PS II). Fluorescence is now used routinely in agricultural and biological research where many measured and calculated parameters are used as biomarkers or indicators of plant tolerance to different abiotic and biotic stress. This has been made possible by the rapid development of new fluorometers. Most of these instruments are mainly based on two different operational principles for the measurement of variable chlorophyll *a* fluorescence: (1) pulse-amplitude-modulated (PAM) excitation followed by measurement of prompt fluorescence and (2) a strong continuous actinic excitation leading to prompt fluorescence. In addition to fluorometers, other instruments have been developed to measure other signals, such as delayed fluorescence, originating mainly from PS II, and light-induced absorbance changes due to the photo-oxidation of the reaction center P700 of PS I, measured as absorption decrease (photobleaching) at about 705 nm, or increase at 820 nm. This chapter includes technical and theoretical basis of newly developed instruments that allow for simultaneous measurement of the prompt fluorescence (PF) and the delayed fluorescence (DF) as well as some other

parameters. Special emphasis is given here to a description of comparative measurements on PF and DF. Since DF is much less used and less known than PF, it is discussed in greater details; it has great potential to provide useful, and qualitatively new information on the back reactions of PS II electron transfer.
This chapter, which also deals with the history of fluorometers, is dedicated to David Walker (1928–2012), who was a pioneer in the field of photosynthesis and chlorophyll fluorescence.

Keywords: delayed fluorescence, fluorometers, Photosystem II, prompt fluorescence

Abbreviations

AL — actinic light
Chl — chlorophyll
Cyt — cytochrome
DF — delayed fluorescence
DLE — delayed light emission
FDP — Fluorescence Detector Probe
F_0 and F_M — minimum and maximum fluorescence intensity, respectively, measured in dark-adapted samples
F'_0, F'_S and F'_M — levels of the fluorescence intensity measured after light adaptation: initial, steady state and maximum fluorescence intensity, respectively
I_1–I_2–I_3... — phases of the DF induction curve
LED — light emitting diode
M-PEA — Multi-Function Plant Efficiency Analyser (Hansatech Instruments Company)
MR — reflectanceof modulated light at 820 nm
NADP — Nicotinamide adenine dinucleotide phosphate
NPQ — non — photochemical quenching of the excited state of Chl *a*, or of Chl *a* fluorescence
O, J, I, P, S, M and T — transient steps in the Chl *a* fluorescence induction curve, appearing between the initial minimal level, and the terminal steady state level; here, O is for the minimal level (Fo), P is for the peak (maximum level, F_M), J and I are intermediate levels between "O" and "P", S is for semi-steady state; and T is for terminal steady state (F_T) obtained during continuous illumination in a dark-adapted photosynthetic sample
P680 and P700 — photochemically active chlorophyll *a* molecule of PS I and PS II reaction centers, which have one of their absorption maxima at 680 nm and 700 nm
PAM fluorometer — a fluorometer based on the pulse amplitude-modulation of measuring light beam
PF — prompt fluorescence
Pheo — pheophytin
PQ — plastoquinone
PQH_2 — plastoquinol
PS I and PS II — Photosystems I and II
Q_A and Q_B — primaryand secondary plastoquinone electron acceptors of PS II;
RC, reaction center;
SP — saturating (light) pulse
S-states S_0, S_1, S_2, S_3, and S_4 — different redox states of the oxygene volving complex
UV — ultraviolet
V_t — variable PF
Z, Y_Z Tyr — tyrosine-161 on the D1 protein of PS II
ΔpH — proton trans-thylakoid gradient
$\Delta\Psi$ — membrane potential

Introduction

History of light emission[3]. In 1565, Nicolás Monardes, a Spanish physician and botanist, published his *Historia medicinal de las cosas que se traen de nuestras Indias Occidentales* in which he observed a «*bluish opalescence*» in the water infusion from the wood of a small Mexican tree. A flemish botanist, Charles de L'Écluse (1526–1609), provided a Latin translation of Monardes' work in 1574, in which we read that the wood used by Monardes was *Lignum nephriticum* (kidney wood), which was very popular then for the treatment of kidney diseases. A translation by an Englishman, John Frampton, in 1577, interestingly mentions a «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». In his 1646 book *Ars Magna Lucis et Umbrae* Athanasius, Kircher (1601–1608), a German Jesuit priest, wrote about «light passing through an aqueous infusion of this wood which made it appear more yellow whereas white light reflected from the solution appeared blue». In 1664, Robert Boyle experimented with this system more precisely. He discovered 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. Moreover, acid seemed to abolish the color and alkali restored it — a pH effect. (W. E. Safford showed in 1915 that the Mexican *L. nephriticum* is taxonomically *Eynsemhardtia polystachia*.)

Then, in Bologna, there was the discovery of light from a stone (subsequently named laparis solaris), which emitted purple-blue light in the dark after it had been baked. This discovery was made, in 1603, by a shoemaker, Vincenzo Casciarolo, who dreamt of producing gold. The famous Galileo Galilei (1612) got into 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 discovered using his naked eye that a brilliant red color was observed from the side when a beam of bright sunlight passed through an alcoholic extract of laurel leaves (see Govindjee (1995)). This solution must have contained chlorophyll (Chl), which had been so named by Pelletier and Caventou (1818), who considered this effect to be due to «dispersion». Soon thereafter, John Herschel (1845) termed this phenomenon «epipolic dispersion» after making the first observation of fluorescence, a beautiful blue light from quinine sulphate (a component of what is in «tonic water»). His paper was titled: *On a case of superficial colour presented by a homogeneous liquid internally colourless*. The author was listed as: *Sir John Frederick William Herschel, Bart., K. H., F. R. S.* (*This paper was received on January* 28, 1845, *and read on February* 13, 1845.)

The aforementioned Bolognian stone is known to be impure barium sulphide. Interestingly, Edmond Becquerel reported in 1842 that excitation of calcium sulphate by ultraviolet (UV) light produced a bluish emission, and that the emission occurred at a wavelength longer than that of

[3]We note that our presentation here is based on a summary of the early history of light emission in nature by Beniamino Barbieri, which was done with the help of David Jameson; it dealt with discoveries on light emission in living systems, and is available at: http://www.fluorescence-foundation.org/lectures/madrid 2010/lecture1.pdf (accessed on June 12, 2012). In addition, E. Newton Harvey (1957) has also published on the history of light emission starting from the earliest times (BCE) and ending in the last decades of the 19th century; this is also available online at: http://www.archive.org/stream/historyoflumines00harv/history oflumines 00harv_djvu.txt. It includes the history of all sorts of light emission, many being very dim indeed: glow of phosphorus; chemiluminescence; the phosphorescence of certain solids after being 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. This earlier discussion of light emission also included bioluminescence such as 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. Our chapter does not mention these types of light emission since the focus is given to light emission particularly from chlorophyll *a* in photosynthetic organisms; as a prelude, the early history of research on light emission from plants, as well as from other sources is presented in this chapter.

the incident light. When the British mathematician Sir George G. Stokes discovered that emission spectra shift to longer wavelengths than the absorption spectra, this phenomenon became well known; it was named the «Stokes shift» in honor of its discoverer. (For a biography of Stokes, see http://www.giffordlectures.org/Author.asp?AuthorID=160.) Stokes saw a blue light through a yellowish glass of wine (transmitting > 400 nm) when sunlight filtered through the blue glass of a church window (excitation light, < 400 nm) impinged on a solution of quinine (see Lakowicz (1999)). Stokes later used a prism to obtain different wavelengths of light to illuminate a solution of quinine. However, it was not until the solution was placed in the UV region of the spectrum that the emission was obtained. As mentioned above, this led to the difference between absorption and emission being called the «Stokes shift» since Stokes declared that fluorescence has a longer wavelength than the exciting light.

We also note that Stokes is the one who had also coined the term *fluorescence* for this phenomenon making reference to the blue-white fluorescent mineral fluorite (fluorspar). He was also perhaps the first to have observed both phycobilin and Chl *a* fluorescence in fresh red algae (Askenasy, 1867). In addition, Stokes also suggested in his lecture: *On the application of the optical properties to detection and discrimination of organic substances*..., in 1864, that fluorescence should be used as an analytical tool. His extensive treatise «On the Change of Refrangibility of Light» used the term «dispersive reflection» to describe light emission in quinine sulphate. One of his other papers had the same title: *On the change of refrangibility of light*, and the author was listed as G. G. Stokes, M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge. The paper was received on May 11, 1852; and read on May 27, 1852, before the Royal Society.

The term *Luminescenz*, implying light, was first used in 1888 by Eilhard Wiedemann, a German physicist and a historian of science; it described «*all those phenomena of light, which are not solely conditioned by the rise in temperature*». That all liquids and solids emit radiation at shorter wavelengths as they are heated above absolute zero is well-known: the material becomes red hot and then white hot. This «hot light» has a different physical basis than that of luminescence, i. e. «cold light». Light from the sun, flashlight (or «torch» run on batteries), candles on festive occasions, oil lamps, gas burners, and electric light bulbs as well as fluorescent tubes are all sources of light in our everyday lives. For further discussion, see Harvey (1957).

Fluorescence

Fluorescence, when used in a generic sense, 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 (e. g. absorption of light, sound or pressure), mechanical (friction), or a chemical mechanism. The phenomenon termed photoluminescence is the generation of luminescence through excitation of a molecule by light (UV or visible); it 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, which absorb light at a particular wavelength, and subsequently emit light after a brief interval, usually at longer wavelengths. Information on the rate constant of this process is given by the lifetime of the fluorescence (see e. g., Noormnrarm and Clegg (2009)). Phosphorescence occurs in a similar manner to fluorescence; however, it has a much longer excited state lifetime (for related information, see http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorhome.html). Phosphorescence originates in triplet states, whereas fluorescence originates from the lowest singlet excited states (see discussion in Lakowicz (1983)). Our discussion in this chapter focuses on fluorescence and delayed fluorescence (see below). For phosphorescence in photosynthetic systems, see Krasnovsky (1982) and Nevrov et al. (2011). For an understanding of fluorescence, see Valeur and Berberan-Santos (2012).

As mentioned above, we deal here with prompt fluorescence, for short PF (i. e. light emission when an electron in the first excited singlet state drops to the ground state), and delayed fluorescence, DF (also known as delayed light emission, DLE), when the first singlet excited state is reached via a recombination of charges, not by light absorption. For earlier reviews dealing with both PF and DF, see Govindjee and Jursinic (1979); for DF, see Lavorel (1975); and for PF, see Butler (1966).

Prompt Fluorescence (PF) from Chlorophyll *a*

We have already alluded to the discoveries of Brewster and Stokes (see above). One of the earliest clues of the relationship of fluorescence to photosynthesis was given by Müller (1874), although there were insufficient controls in his experiments and his ideas on the basic concepts of light were erroneous; he did, however, notice that the red Chl *a* fluorescence of a living green leaf had a much weaker signal than that from a Chl solution (Govindjee, 1995). Kautsky and Hirsch (1931) observed, with their eyes, Chl *a* fluorescence to rise rapidly to a maximum level, then decline and finally reach a steady level, all within a matter of minutes. They considered that the rising portion of the curve reflected the primary photochemical reaction of photosynthesis, as it was unaffected by temperature (0 and 30 °C). The decline appeared to be inversely correlated with the increase in the rate of CO_2 assimilation, measured earlier by Warburg (1920); this suggested to Kautsky and Hirsch (1931) that less Chl fluorescence is seen when more chemical energy is produced from photons (*complementary relationship*). Many investigators have used many available instruments to perform Chl fluorescence transient measurements on photosynthetic samples showing many inflection points, which have been labelled, in the past, as A, B, C, D, and E, or Dl, Ml, D2, and 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 Figure 1 for OJIPSMT chlorophyll *a* fluorescence transients, obtained from pea leaves under two different light conditions.)

This nomenclature has a history. 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 Jr and Govindjee (1969a, 1969b): 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, 1968b) called it SMT, where S is the semi-steady state, M is the maximum and T is the terminal steady state. Partly based on terminology by Bannister and Rice (1968), the several additional waves sometime seen in between some of the states mentioned have been labelled as, e. g., S1, M1, S2, and M2 (Yamagishi et al., 1978). On the other hand, Neubauer and Schreiber (1987) and Schreiber and Neubauer (1987) used O-I_1-I_2-P for the fast transient when they saw two instead of one inflection between the O and the P levels; the O-I_1-I_2-P nomenclature was replaced by the OJIP nomenclature of Strasser and Govindjee (1992) for convenience in typing. However, for further discussion, see Schreiber and Krieger (1996). 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 in addition to the clearly visible steps in the OJIP fluorescence rise; the nomenclature of these fluorescence bands between Fo (= O) and Fp (= 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, Ft has been regularly used for fluorescence at time «t». This chapter does not discuss the L, K, H and G levels. (There are several papers available on the application of fluorescence to stress conditions; for a simple and a quick application of fluorescence to stress conditions, see Lichtenthaler and Rinderle, 1988.)

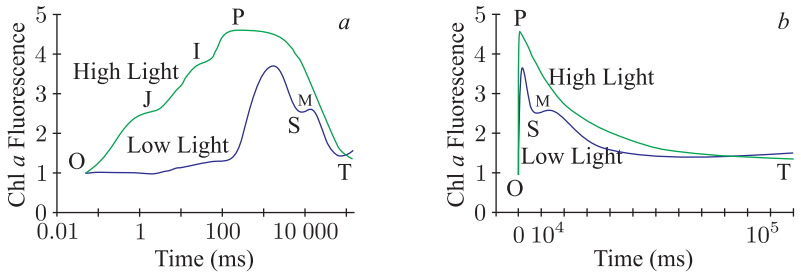

Fig. 1. Typical chlorophyll *a* fluorescence induction transients (Kautsky curves), at two different excitation light intensities. Sample: a 20 min dark-adapted pea leaf. **Left:** on a logarithmic time scale; **Right:** on a linear time scale. Wavelength of excitation: 650 nm. Excitation light intensity for curves labelled **low light** was ~ 30 micromol photons $m^{-2}s^{-1}$ at the leaf surface; for high light, $\sim 3\,000$ micromol photons $m^{-2}s^{-1}$ 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_O, where F_O is the initial fluorescence (arbitrarily taken at 50 μs) and F is fluorescence at any other time; thus, it is in relative or arbitrary units. The initial low fluorescence intensity is interpreted as being due to quenching of fluorescence by highly efficient PS II photochemistry. The rise in fluorescence is due mainly to reduction of Q_A, an electron acceptor of PS II (for recent reviews, see Stirbet and Govindjee 2011, 2012). The slow fluorescence transient (P-S-M-T) is due to several reasons including (i) a re-oxidation of reduced Q_A, (ii) quenching by transmembrane ΔpH, and (iii) transition from high fluorescent state I to low fluorescent state II, and vice versa (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). Reproduced with permission

The first quantitative complementary relationship between fluorescence and photosynthesis (i. e., CO_2 assimilation, or O_2 evolution) was obtained by MacAlister and Myers (1940). 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! Duysens and Sweers (1963) provided a major concept by proposing the «Q» hypothesis, i. e., fluorescence is low when Q (now called Q_A) is in its oxidized state, and fluorescence is high when Q_A is reduced to Q_A^-. Further, Papageorgiou and Govindjee (1968a, 1968b) and Mohanty et al. (1971) showed a parallel increase in fluorescence and O_2 evolution during the slow S to M phase, and constancy of O_2 evolution during the MT decline. Thus, only under certain experimental conditions can this anti-parallel relationship between fluorescence and photosynthesis be observed. There are at least four pathways for the de-excitation of an excited state: (1) **photochemistry**; (2) **fluorescence**; (3) **heat** and (4) **excitation energy migration** to neighbouring pigment complexes (cf. Govindjee 2004). This last process is of special importance. For example, Strasser and Butler (1977a, b) showed that excitation energy transfer from photosystem II (PS II) to photosystem I (PS I) led to an appreciable amount (over 50 %) of PS I activity (P700 photo-oxidation) at 77 K, when light was absorbed originally in PS II.

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

Parallel measurements on the time course of oxygen evolution and chlorophyll fluorescence in the green alga *Chlorella*, and in a cyanobacterium *Anacystis*, have been presented by Bannister and Rice (1968) and Papageorgiou and Govindjee (1968a, 1968b). Further, Walker et al. (1983) showed parallel measurements on the oscillations of oxygen evolution and of Chl fluorescence in spinach leaf pieces, observing a clear anti-parallel relationship between $d[O_2)/dt$ and fluorescence curves. Further, Strasser (1985, 1986) showed that fluorescence and oxygen evolution have similar

trends (e. g. rise and decays or oscillations), but with different changes in the rate constants. However, only when the heat loss is negligible and constant, an anti-parallel relationship between fluorescence and photochemistry is possible. For a historical discussion, see reviews by Govindjee (1995, 2004).

As noted above, the light emitted at the time of the de-excitation of the first excited state of Chl has two components: prompt fluorescence (PF), and delayed fluorescence (DF); in general, the DF component is only a very small part of this emission (however, see Klimov et al., 1978). Approximately 5 ns after the light has been switched off, PF emission is practically extinguished (see reviews by Jursinic 1986; and Krause and Weis 1991); its intensity decays in a polyphasic manner, with characteristic lifetimes that range from several ps to several ns (see e. g., Miloslavina et al., 2006). Delayed fluorescence (DF) is light emission by Chl molecules after the prompt fluorescence has decayed. It decays in the dark, also in a polyphasic manner, and has components in very different time domains: in time ranges of *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 *minutes to hours* (Hideg et al., 1990). However, an involvement of lipid peroxidation could also take place in the long time range: Chls emit photons as a consequence of steps beginning with lipid peroxidation, which, in turn, is initiated by reactive oxygen species (Hideg et al., 1991). Prasad and Pospíšil (2011) have described a detailed mechanism of photon emission, from the chlorophylls, under *in vivo* conditions. They have 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.

Mechanisms of PF are not described here in this chapter; these can be found elsewhere in available books on PF (see e. g. Govindjee et al. 1986; Papageorgiou and Govindjee 2004). However, we will discuss DF in some details since it is much less used and much less known than PF.

Delayed Fluorescence (DF) from Chlorophyll *a*

Delayed fluorescence, DF (also called DLE, delayed light emission), is a lower intensity longer-lived light emission than PF. Strehler and Arnold (1951) (also see Strehler 1951) discovered DF, 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). Since DF has an emission spectrum almost identical to that of Chl fluorescence, it must originate in the de-excitation of excited Chl *a* (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 indicates that in both cases the photon release is a result of the radiative deactivation of the singlet excited state of the PS II antenna Chl *a* (Krause and Weis, 1991; Lang and Lichtenthaler, 1991). Indeed, it is well established that DF in plants, algae and cyanobacteria originates mainly in PS II, since it is absent in algal mutants, which lack PS II (Bertsch et al., 1967; Lavorel, 1969; Haug et al., 1972; Bennoun and Béal, 1997; Turzó et al., 1998). Further: **(1)** the action spectra for PS II 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 PS II than in PS I-enriched particles (Lurie et al., 1972; Vernon et al., 1972; Itoh and Murata, 1973; Gasanov and Govindjee, 1974). The yield from the DF in PS I (Shuvalov, 1976) is significantly much lower than from PS II.

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

Mechanism of Delayed Fluorescence

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 PS II electron donor ^{1}P680* and Chl antenna of PS II; (2) the radical pair P680$^+$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 different opinion on P680$^+$Pheo$^-$ generation, see e. g. Shelaev et al. 2011; and for a general review on PS II, see Govindjee et al. 2010). The excited antenna Chl molecules, formed as a result of this reversal of charge separation, emit the DF quanta, followed by fast excitation energy transfer from ^{1}P680* to Chl antenna (Dau and Sauer, 1996; Grabolle and Dau, 2005):

$$^1\mathrm{P680} * \mathrm{Pheo} \leftrightarrows \mathrm{P680}^+\mathrm{Pheo}^-. \quad (1)$$

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

A decrease in the number of charge couples (precursors of excited states of ^{1}P680* and, thus, of emitted DF quanta) or changes in the DF quantum yield lead to decreases in DF intensity. Usually, the back reactions in PS II are several orders of magnitude slower than the forward reactions and their contribution to the DF decay rate could be neglected (Lavorel, 1975). The back reactions determine DF decay when the forward electron transfer is stopped (e. g., when the PQ pool is fully reduced or when the PS II herbicides are present blocking electron flow).

There are three types of reactions that determine the kinetics of DF dark relaxation (cf. Lavorel 1975; Goltsev et al. 2009):

(1) The main mechanism of DF decay in the micro- and sub-millisecond time range is *leakage* type reactions — i. e., when the decrease in DF precursors is mainly the result of the disappearance of the negative or positive charges from the radical pair P680$^+$Pheo$^-$, as e. g. by: **(a)** reoxidation of Pheo$^-$ by Q_A, or **(b)** reduction of P680$^+$ by the electron donor Z (i. e., Y_Z).

(2) The *deactivation* type reactions — i. e., when DF precursors decrease due to redox reactions within the charge pair (i. e., by recombination of charges within P680$^+$Pheo$^-$) (cf. Lavorel, 1975; with Klimov et al., 1978). However, the formation of the excited state of P680 (^{1}P680*), and thus to DF can be produced by a small part of this recombination reaction. The «deactivation» of DF precursors (P680$^+Q_A^-$, Z$^+$P680Q_A^- or S_2ZP680$Q_AQ_B^-$) through backward electron transport reactions, followed by charge recombination, contributes to the slower DF components (milliseconds and longer). [The DF intensity decreases because of the disappearance of the separated charge couples in both «leakage» and the «deactivation» cases.]

(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, $\Delta\Psi$).

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

$$L(t) = \sum_t L_i e^{-t/\tau_i}, \quad (2)$$

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. The deactivation type of DF can also be described using this model when the separated charges remain in the same protein complex, as is the case, for example, of the PS II state: $Z^+P680Q_A^-$, where Z (i. e., Y_Z) is tyrosine-161 on the D1 protein of PS II.

$P680^+Pheo^-$ is the only *direct* precursor, which recombines and forms the excited state of Chl in the PS II reaction center. The other PS II redox states which are the major DF precursors, and, thus, responsible for DF generation are: $P680^+Q_A^-$, $Z^+Q_A^-$, $Z^+Q_B^-$ and S_iZQ_B, where Q_B is the second plastoquinone electron acceptor of PS II. The backward electron transfer, the formation of $P680^+Pheo^-$ and their recombination produce routes for DF generation. The DF emitted in the microsecond and the millisecond time domain is mostly related to backward electron transfer and the recombination of charges in $P680^+Q_A^-$ and $Z^+Q_A^-$ states of PS II (see Figure 2 for an energy level diagram which explains DF).

The kinetics of DF dark decay depend on the rates of the following three redox reactions, for example, when the dark decay originates from PS II in the $Z^+Q_A^-$ state: **(a)** reoxidation of

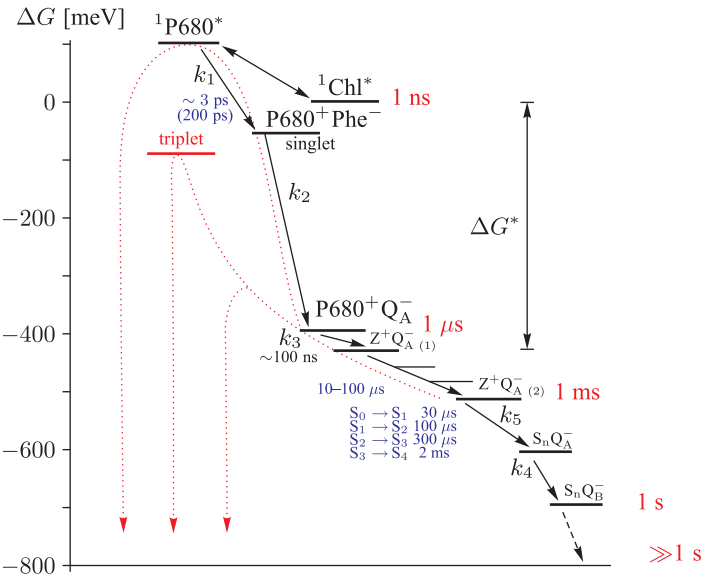

Fig. 2. Energy level diagram for the Photosystem (PS) II states participating in delayed fluorescence (DF) generation. ΔG values (in meV) (on the left ordinate) indicate estimated Gibbs free energy levels of PS II redox states participating in DF generation. ^{3}P680 is shown simply by «triplet» in the diagram. *For this diagram, the ΔG value of the excited state of antenna 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_is are the rate constants of electron transfer (ET) reactions within PS II: k_1 is for primary charge separation in (singlet) excited PS II 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 $P680^+$; $P680^+Pheo^-$ is the PS II primary radical pair. The rate constants k_3 and k_5 are for ET reactions on the electron donor side of PS II, and k_4 is for ET reaction on the acceptor side of PS II. The formation of the (initial) state $Z^+Q_{A(1)}^-$ 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 $Z^+Q_{A(2)}^-$ 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 PS II. The values in red are lifetimes of the back reactions. Modified by one of the co-authors (VG), from Grabolle and Dau (2005), Dau and Zaharieva (2009), and Dau et al. (2012)

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 the state $S_iZ^+Q_A^-$ to the state $S_{i+1}ZQ_A^-$; and **(c)** charge recombination between Z^+ and Q_A^-. When the direct redox reactions are interrupted by a physical or a chemical treatment, the last reaction determines the DF decay rate.

Measurements of Chlorophyll Fluorescence

Fluorometric method

When a chlorophyll molecule absorbs photons in the blue to the red region of the spectrum, fluorescence occurs in the red region of the spectrum: the transfer of an electron from the ground state to the excited state of the molecule is caused by light absorption; as the electron returns from the first excited state to the ground state, the molecule rapidly emits light, the prompt fluorescence (for basics and principles, see Clayton 1971; see Lakowicz 1983, for details). The relationship between absorbed and emitted photons at different wavelengths are characterized by measurements of excitation (action) and emission spectra. Fluorescence measurementis a precise, relatively inexpensive, and easily mastered quantitative, analytical technique. Conventional portable fluorometers can measure remotely from a few millimetres (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 and thus can be used to study diurnal (Sweeney et al., 1979), as well seasonal acclimation of PS II (Porcar-Castell, 2008).

Molecular fluorescence for qualitative analysis and semi-quantitative analysis has been used since the early to mid-1800s, but more accurate quantitative methods appeared 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 fluorescence was discovered almost 200 years after phosphorescence, qualitative and quantitative applications of molecular phosphorescence were not given much attention until fluorescence instrumentation had developed (Valeur, 2001; Valeur and Barberan-Santos, 2012).

Selected examples

Physiological and low temperature Chl *a* fluorescence measurements (e. g., of kinetics; action (excitation) and emission spectra; depolarization; and lifetimes) have provided critical information on almost every aspect of light absorption and conversion process in photosynthesis, with special relevance to our understanding of: (1) excitation energy migration within the antenna and to the reaction centers, (2) the energetic connectivity between the antennas and the reaction centres, (3) the primary photochemistry and the secondary electron transport associated with the primary reactions. Several reviews on the use of fluorescence techniques in photosynthesis are available, e. g., those 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., Stribet and Govindjee (2011, 2012), and Papageorgiou and Govindjee (2011), and references therein. Information on the measurement and questions related to non-photochemical quenching (NPQ),of the excited state of Chl, can be obtained by using the pulse-amplitude-modulation (PAM) method, shown in Figure 3 (see Schreiber 2004).

Chlorophyll fluorescence measurements are a trusted tool not only for the study of the structure and function of photosynthetic apparatus (see e. g., Govindjee et al., 1976; Eaton-Rye and Govindjee, 1988a, 1988b; Allakhverdiev et al., 1994; Strasser and Strasser, 1995; Bukhov and Carpentier, 2000; Bukhov et al., 2001; Antal et al., 2007; Baker, 2008; Allakhverdiev, 2011; Bussotti et al., 2011a; Garcia-Mendoza et al., 2011; Matsubara et al., 2011; Brestič et al., 2012;

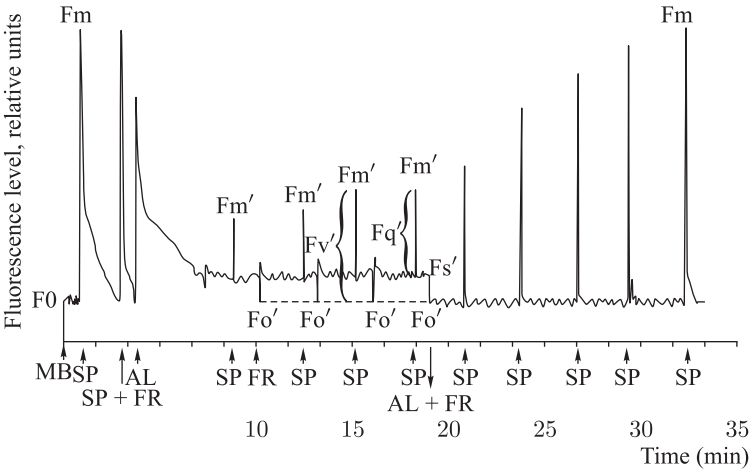

Fig. 3. A schematic tracing of Chl *a* fluorescence measurement, using a pulse-amplitude-modulated (PAM) fluorometer (see e. g., Schreiber, 2004). In this method, a dark-adapted photosynthetic sample is first exposed to a very weak measuring beam (MB) to obtain the initial fluorescence level Fo (the «O» level). Then, a saturating light pulse (SP) is applied to take the sample to the fluorescence maximum Fm, which slowly returns to F_O. This return can be accelerated by applying a far-red (FR) light to the sample, absorbed mostly in Photosystem I (PS I); the new *Fo* level is called Fo′, whereas Fs′ refers to steady state fluorescence in light. Other symbols Fv′ and Fq′ are defined as (Fm′−Fo′), and, (Fm′−Fs′), 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 Fm′ (< Fm). After switching off the AL, a progressive recovery of the maximum fluorescence level (Fm) is obtained after a SP is given; this reflects the relaxation of the non-photochemical quenching (NPQ) of the excited state of Chl. The full recovery to the Fm level indicates that no RCIIs have been irreversibly damaged during the light period. Source of the figure and part of its legend is from Henriques (2009)

Goltsev et al., 2012), but also in several other areas, e. g., plant breeding (Baker and Rosenqvist, 2004; Kalaji and Guo, 2008; Kalaji and Pietkiewicz, 2004); seed vigour and seed quality (Jalink et al., 1998; Dell'Aquila et al., 2002; Konstantinova et al., 2002); fruit quality and in controlling the post-harvest processing of fruits and vegetables (Merz et al., 1996; Nedbal et al., 2000a). Further, fluorescence has also been used to monitor 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, 2011b, 2012; Kościelniak et al., 2011), climate change (Ashraf and Harris, 2004), urban conditions (Swoczyna et al., 2010a, 2010b), environment and pollution (Croisetiere et al., 2001; Bussotti et al., 2005; Kalaji and Loboda, 2007; Romanowska-Duda et al., 2010; Tuba et al., 2010; Bussotti et al., 2011b), sports field heterogeneity and physiological state (Lejealle et al., 2010; Beard, 2002), and algal blooms and water quality (Gorbunov et al., 1999; Seppälä et al., 1999; Romanowska-Duda et al., 2005; Antal et al., 2009). Specific nutrient deficiency in plants can also be identified with this technique (www.fluorimetrie.com [this site is in French, but an English translation is also available]). Recently, NASA (National Aeronautic Space Agency, of USA) produced a Space Fluorometer (System) in order to develop a first-of-its-kind fluorescence map of the world's plants: http://daac.gsfc.nasa.gov/oceancolor/scifocus/ocean color/warming.shtml).

We now discuss below a wide range of information related to the progress of instrumentation development. The following provides some of the advantages and features of some of the latest available fluorometers and briefly provides relevant technical background for the same.

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

This section provides a perspective on the evolution of various tools and instruments in chronological order.

The Kautsky Effect: Observation by the naked eye

Kautsky and Hirsch's 1931 observations with the naked eye have been mentioned above. At the time of this observations of variable Chl*a* fluorescence in plants, Hans W. Kautsky (1891–1966) was an assistant professor at the *Chemisches Institut der Universität Heidelberg*, Germany. (He was at that time investigating the action and properties of active singlet oxygen in photosensitisation processes.) Kautsky and A. 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 entitled «New experiments on carbon dioxide assimilation». Observed with the authors' naked eye, the time course of Chl*a* fluorescence was qualitatively correlated with the time course of CO_2 assimilation, published earlier by Otto Warburg (1920). (See above, and Govindjee (1995) for an historical account.) Further, Kautsky was the first to suggest that the singlet oxygen quenches fluorescence during CO_2 assimilation. After quite a long period when his work was not noticed, his research on the role of singlet oxygen in photosynthesis was finally recognized in 1964. The following web site is dedicated to Kautsky's work on fluorescence and other areas: http://www.fluoromatics.com/kautsky_effect.php.

Observations and measurements by laboratory instruments

E. Newton Harvey in 1957 wrote a detailed history of luminescence (see Harvey 1957). As noted earlier in a footnote, it is available at: http://www.archive.org/stream/historyoflumines00harv/historyoflumines00harv_djvu.txt.

As described by Harvey (1957), a device in which materials were exposed to sunlight, and then examined quickly in the dark, was produced by Beccari (1744); with it he was able to detect phosphorescence which lasted several seconds or even tenths of a second. The construction of the first phosphoroscope was pioneered by Edmond Becquerel (1858) more than 100 years later; this device had allowed him to measure the decay times of phosphorescence. In 1888, E. Wiedemann built a phosphoroscope, which shortened the time of the first measurements down to a few microseconds; this work also led to the information that the lifetime of fluorescence of pigments/chromophores was even much shorter than microseconds; we now know that it is in nanosecond to picosecond time scale.

During the early part of the 20th century, (prompt) fluorescence was observed under microscopes by many different scientists. In fact, when August Köhler and Carl Reichert carried out microscopy studies under ultraviolet light, they initially considered fluorescence as a «nuisance». The very first fluorescence microscopes were produced during 1911–1913, in the laboratories of Otto Heimstädt and Heinrich Lehmann. Gaviola designed the very first instrument for measurements on the lifetime of fluorescence, in 1926, basing his instrument on the principle of the phase shift of fluorescence from that of the exciting light. The first photoelectric fluorometer was built, just two years later, by E. Jette and W. West (1928). The other method, the direct flash method, of measuring lifetime of fluorescence uses a flash of excitation light to excite the sample, followed by measurement of the decay of fluorescence in the dark with a weak measuring light. Chl *a in vivo* fluorometry emerged as a major method in photosynthesis research after the invention and improvement of photocells and photomultipliers during the 1930–1950s (see chapters in Papageorgiou and Govindjee, 2004, for the detailed use of fluorescence in photosynthetic systems).

The first commercial fluorescence instrument was produced, during World War II, by the Coleman Company. However, its use, along with that of the Beckman DU absorption spectrophotometer, was restricted by the US military until the end of World War II. Moss and Loomis (1952) constructed their own absorption spectrophotometer and were some of the first to measure absorption, transmission and reflection of leaves in plants as well as in algae. For a review, see Carter and Knapp (2001). Two companies developed spectrofluorometers between 1955–1956: the Aminco-Bowman (Silver Spring, Maryland, USA) and Farrand Optical Company (Walhalla, New York, USA) (see e. g., (Bowman et al., 1955)). In contrast to steady state measurements, Steve Brody, in 1957, at the University of Illinois at Urbana-Champaign, Illinois (USA) was the first to construct a device to measure fluorescence lifetimes in several photosynthetic samples, using direct flash method (Brody, 1957). A phase method to measure the lifetime of fluorescence in the green alga *Chlorella* was developed the same year in Russia (see Dmitrievsky et al. 1957).

We mention here the name of Alexander Jablonski (1898–1980), who is known as «the father of fluorescence spectroscopy»; he had introduced what is known as the Jablonski Energy Diagram, which is used to explain absorption and emission spectra as well as the paths taken by molecules which lead to prompt fluorescence, delayed fluorescence and phosphorescence. The energy level diagram is now called the Perrin – Jablonski diagram since this diagram had followed the earlier pioneering work of F. Perrin (see discussion in Valeur and Brochon (eds.), 2001).

In 1951, Bernard Strehler and William Arnold invented the first apparatus to measure DF in photosynthetic systems (Fig. 4). Their experiments strongly suggested that delayed light emission 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.

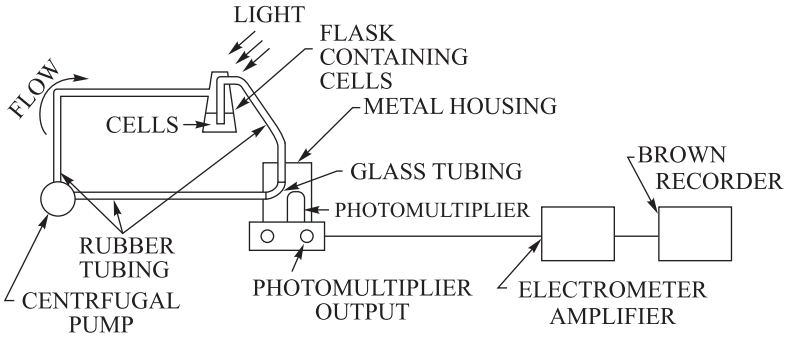

Fig. 4. A schematic diagram of an apparatus which was used to discover and measure delayed fluorescence, DF (also called delayed light emission, DLE) from suspensions of green algae (cells). In experiments on the measurement of DF, cells are illuminated at one time and at one place. Using a flow system, they are then moved to a place in darkness in front of a photomultiplier. All parts of this 1951 instrument are clearly labelled in the diagram. Source: Strehler and Arnold (1951)

Many more laboratory fluorometers were constructed during the 1960s, and used in photosynthesis research; one such instrument was 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 early set-up, see Shimony et al. 1967). Also see Butler (1966) for fluorescence instruments used by Warren Butler and his associates.

During the 1970s and 1980s, many home-made instruments/systems were developed by several scientists. Some examples are given below. In addition to the use of fluorometers alone, other instruments were developed by one of the authors (Reto Strasser) to measure different signals originating from PS II, such as oxygen evolution and absorption changes of Hill-reagents (artificial electron acceptors) (Strasser, 1973a). Strasser (1973b) studied the correlation

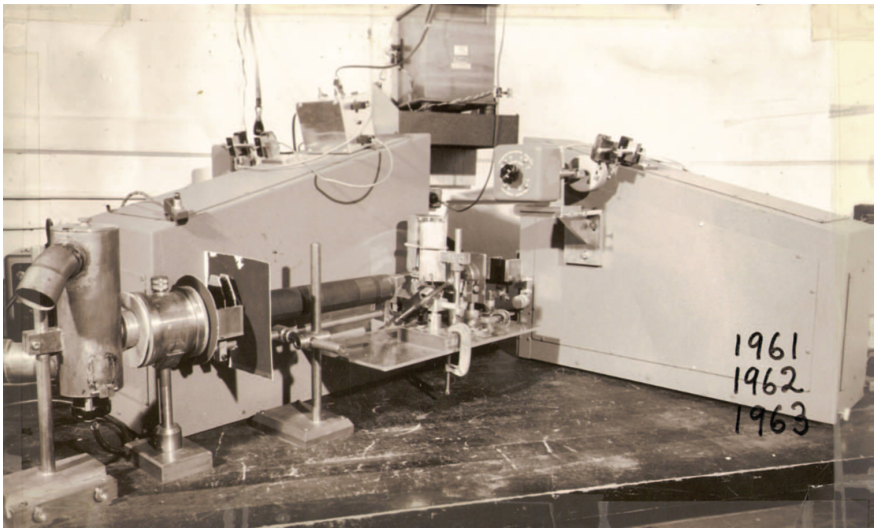

Fig. 5. A photograph of the spectrofluorometer built by Govindjee and Jobie D. Spencer in 1960 used from 1960–1963 for many discoveries including that of the two light effect in chlorophyll *a* fluorescence (Govindjee et al., 1960), and the discovery of a new emission band at ~ 693 nm when photosynthesis was saturated (Krey and Govindjee, 1963); this instrument used two large Bausch and Lomb monochromators (see the sloping large units on the right and the left) for excitation and emission. When the 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 the box at the top), and later using a Brown chart recorder. A diagram of this instrument was published in Shimony et al. (1967). Photo by Govindjee

of simultaneously measured variable fluorescence, DF and oxygen evolution in leaves using this set-up, just when the photosynthetic apparatus was initiated to begin functioning. The induction of PS II activity by measuring the induction of a variable part of the fluorescence emission in flashed bean leaves exposed to weak green light was reported by Strasser and Sironval (1973). Combined measurements of absorption, reflection, prompt and delayed fluorescence emission in flashed leaves were published by Strasser (1974) soon thereafter. With improved methods and instrumentation, quantitative parallel measurements on oxygen evolution burst and variable fluorescence in leaves were provided by Strasser and Sironval (1974). These multi-parameter measurements were made possible because of the use of the custom-made light-guide fibre optics, which had three arms. During 1980s, David Walker and his coworkers made parallel measurements on oxygen evolution and chlorophyll *a* fluorescence (Delieu and Walker (1983), Walker and Osmond (1986), and Walker (1987)). An interesting behavioural pattern was observed by Strasser (1986) when he compared oxygen evolution with DF and PF: he observed both anti-parallel as well as parallel behaviour between oxygen evolution and light emission. The instrument Strasser built with Sironval in Belgium (see Fig. 6) was transported to the laboratory of Warren Butler in the USA, where it was rebuilt and modified for excitation energy transfer measurements in flashed bean leaves, from the core antenna of PS II to PS I (Satoh et al., 1976). The kinetics of absorbance decrease at 705 nm (due to P700 photo-oxidation) was measured for PS I activity.

Some details of the instrument used by Satoh et al. (1976), in Butler's lab, should be mentioned, who used a home-made system to measure Chl fluorescence at 685 nm: Balzer's 685 nm interference filter, two Toshiba V-R68 cut-off filters, and a S-20 EMI 9558 photomultiplier tube. Further, fluorescence was excited by a Ne-He laser system (300 μW/cm^2). The use of similar instruments for measurements of the photoreduction of NADP (Duysens and Amesz, 1957; cf. with

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 a room filled with oscilloscopes, chart recorders and photomultipliers. These instruments were assembled 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 disc 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 4 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)). The above photo was taken by an unnamed assistant in Butler's lab

that of Mi et al., 2000) should also be noted. This type of measurement enabled Satoh et al. (1976) to study excitation energy transfer from PS II to PS I in chloroplasts (cf. Allakhverdiev et al., 2007b).

We also mention Hansatech Instruments Company (Norfolk, England) with which David Walker (see Dedication) and two of us (HK and RJS) have been associated, as an example of a company involved in producing equipment for fluorescence measurements. When Fluorescence Detector Probe (FDP), associated with a control box in 1983, was designed, it was the first time Hansatech became involved with the measurement of Chl*a* fluorescence. This work 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 to a chart recorder over an extended time base, commensurate with the slow pen response speeds of chart recorders. Hansatech has also developed other instruments, which have, e. g., modulated fluorometers (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 the instruments labelled FMS-1 and FMS-2.)

In the 1990s, a small company «TEST» (Krasnoyarsk, in Russia) developed a fluorometer which provided excitation in the blue (400 nm), blue-green (515 nm) and green (540 nm)

region. It was called the FL3003, and was thus able to separate green algae, blue-green algae (cyanobacteria) and diatoms in phytoplankton samples in natural ponds (Gaevsky et al., 1992). Another instrument — the Photon 8 fluorometer — allowing measurements of DF in various photosynthetic organisms: algal suspensions, isolated chloroplasts, plant leaves, pine needles, and lichens, was also produced by the same company in 1992.

Several research groups had been building, and using phosphoroscopes, for Chl *a* DF measurements in the 1960s. Walter Bertsch produced one of the earliest instruments (Bertsch and Azzi, 1965, Bertsch et al., 1967, 1969). Many labs at this time (late 1960s and beyond), notably that of Louis N. M. Duysens (see e. g., Sonneveld et al. 1980a, 1980b, and references therein) and of Anthony R. Crofts (see e. g., Crofts et al., 1971, and references therein) saw the use of devices for simultaneous measurement of PF and DF. Here, we mention a portable multi-flash kinetic fluorometer, published by Kramer et al. (1990), for the measurement of donor and acceptor reactions of PS II in leaves of intact plants under field conditions.

Venediktov et al. (1969) developed a phosphoroscope to measure DLE in which 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 other instruments, to measure both PF and DF (Fig. 7) (see later for a complete discussion of measurements from this instrument).

There have been several other instruments built around the world to measure PF and DF, and all have provided useful data. Since work published in a non-English language is not often known internationally, we mention here a home-made device which was constructed and used in Poland by Antoni Murkowski and Aleksander Brzóstowicz to measure DF kinetics from leaf samples in the 0.5 s to 20 s range (see Brzóstowicz, 2003; Brzóstowicz et al., 2003; Murkowski, 2002; Murkowski and Prokowski, 2003).

Observations and measurements by portable instruments

Basic photosynthesis research has been greatly influenced by modulated fluorometers featuring mechanical choppers and lock-in amplifiers. In the 1970s, an essential development in the area of Chl fluorescence instrumentation was made possible by the technical progress in electro-photonics (availability of light emitting diodes (LEDs) and fast photodiodes). The first portable fluorometer for field studies was constructed by Ulrich Schreiber et al. (1975). However, it was limited to the recording of dark-light induction curves. Further physiological research by Briantais et al. (1979); Bradbury and Baker (1981); Krause et al. (1982); Horton (1983); and Walker et al. (1983) provided decisive stimulation leading to progress in instrumentation. Chopper-modulated fluorometers were first used to distinguish between 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*, which dealt with their «Pulse-Amplitude-Modulated (PAM) fluorometer» (see data shown in Fig. 3). This instrument had state-of-the-art properties: distinguishing ambient from modulated light, modulated fluorescence excitation and high selectivity of the fluorescence amplifier for the modulated signal (Schreiber and Schliwa, 1987). Schreiber (2004) has reviewed detailed technical specification of such a system; this instrument is commonly used in saturating pulse (SP)-mode for the determination of fluorescence parameters associated with the slow fluorescence changes, including the measurement of the recovery of the initial fluorescence yield after illumination pulses (see Fig. 3; and results obtained with PAM fluorometer by e. g. Demmig-Adams et al. 1996).

One of the most used as well as misused methods in the understanding of the physiology of plants, algae and cyanobacteria has been prompt Chl *a* fluorescence measurements — discussed in this chapter. There are many factors involved in the complex relation between photosynthesis and fluorescence. Logan et al. (2007) have discussed the common errors in the use of chlorophyll

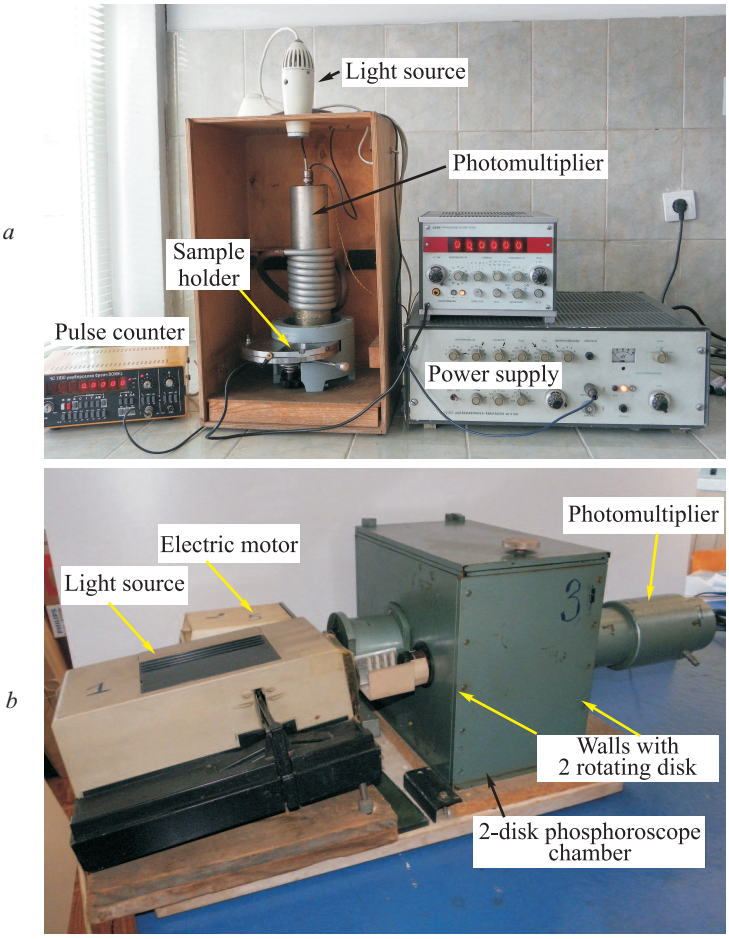

Fig. 7. Delayed Fluorescence instrument, used in the laboratory of one of the authors (VG). Since DF is discussed in greater details in this review, we have also provided details of the DF measuring device. All the major parts of the instrument are labelled on the diagram. *a* — Experimental device for the recording of long-lived (0.5–100 s) DF after the actinic light had been turned off. Detached leaves or chloroplast suspensions (see sample holder) were illuminated for 20 s with light from a 40-W tungsten lamp (top), which passed through two colored glass filters (BG 18, Schott, Mainz, Germany, and CS 5030, Corning, Rochester, NY, USA). The energy of the exciting light was 5 Wm^{-2}. After illumination, the sample was mechanically moved toward the photomultiplier photocathode. Delayed-fluorescence (DF), 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 photo-current modes (Yordanov et al., 1987). *b* — Laboratory–built device for millisecond DF recording. This fluorometer was designed as a two-disc-phosphoroscope of the Becquerel-type (produced at the Moscow University, USSR, now Russia). Photographs were taken by one of the authors (VG)

fluorescence on plants under field conditions. They have advised researchers that before relying on the automatically calculated parameters of the instruments used, the raw data traces must be examined first: more importantly, 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. A critical discussion of relevant problems

dealing with the important question of photoprotection and photoinhibition when plants are exposed to excess light is dealt with in several chapters in Demmig-Adams et al. (2005).

The next generation fluorometers have provided very sensitive measurements on low Chl content samples, and fluorescence from single cells (Küpper et al., 2009). Here, 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). The same PAM technique was also used to measure P700 absorbance (Klughammer and Schreiber, 1998). Klughammer et al. (1990) developed a 16-channel LED-array spectrophotometer for the measurement of time-resolved difference spectra in the 530–600 nm region in order to measure absorbance changes of cytochromes (Cyt *f*, Cyt *b*-563 and Cyt *b*-559). Kolbowski et al. (1990) developed a computer-controlled pulse-modulated system for the analysis of photo-acoustic signals. Reising and Schreiber (1992) used this technique to study pulse-modulated heat release, O_2 evolution and CO_2 uptake associated with stromal alkalization (Schreiber, 2004) and the detection of thermal deactivation processes (Allakhverdiev et al., 1994).

The end of the *Cold War* between USA and the Soviet Union (1991) was important for instrumentation development and distribution. Defence secrets and advanced instruments, which had been kept only for the military were made available for civilian scientific research, creating the era of the portability of instruments in biology and physiology as well.

Two worldwide companies, Hansatech Instruments Ltd. (UK) and Heinz Walz GmbH (Germany; http://www.walz.com), have developed many lab and portable fluorometers to measure Chl *a* fluorescence from intact leaves and from algal suspensions in liquid media in the last 30 years. This development was a result of the long-term cooperation and scientific support of the late David Walker (1928–2012); see Edwards and Heber (2012) and Reto Strasser for Hansatech Company, and Ulrich Schreiber for the Walz Company. Both companies have a very professional set of instruments; this has been possible because of advances in research in using Chl *a* fluorescence during the last three decades. Currently, Photon Systems Instrumentation (for information, see http://www.psi.cz/about-psi/our-company) is another emerging company with the earlier conceptual and technical support of Ladislav Nedbal. Furthermore, there are multi-wavelength kinetic fluorometers (MWKF), based on photodiode array detectors, which provide 3-dimensional fluorescence induction kinetics (F vs λ vs t). See Kaňa et al. (2009, 2012) for further details and applications of this instrument.

A fast repetition rate (FRR) method was devised by Kolber et al. (1998) to measure variable fluorescence; it has been applied to many photosynthetic systems. Further, Kolber et al. (2005) have used a laser-induced fluorescence transient (LIFT) to remotely monitor terrestrial vegetation. Other studies on photosynthesis are using special instruments, with multiple functions. This may result in answers to many important open questions related to specific processes, which influence the quantum yield of Chl *a* fluorescence, and, thus, the quantum yield of photosynthesis. 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*, Cyt *c*-550, Cyt *b*-559), 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. Advanced instruments of this type, such as the Multi-Function Plant Efficiency Analyser (M-PEA), have been produced by Hansatech; details of this instrument are given below.

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

The Multi-Function Plant Efficiency Analyser (M-PEA), from Hansatech Instruments Ltd, is a recent development. (Fig. 8; also see Strasser et al., 2010); it combines 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»). All of the light sources and detectors are combined in the instrument, in the sensor unit itself, and are covered by a quartz window, which 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 PS I. Further, the M-PEA also includes a high sensitivity DF detector, as well as a detector to measure leaf absorptivity (For information on Hasatech products, go to: http://www.hansatech-instruments.com.)

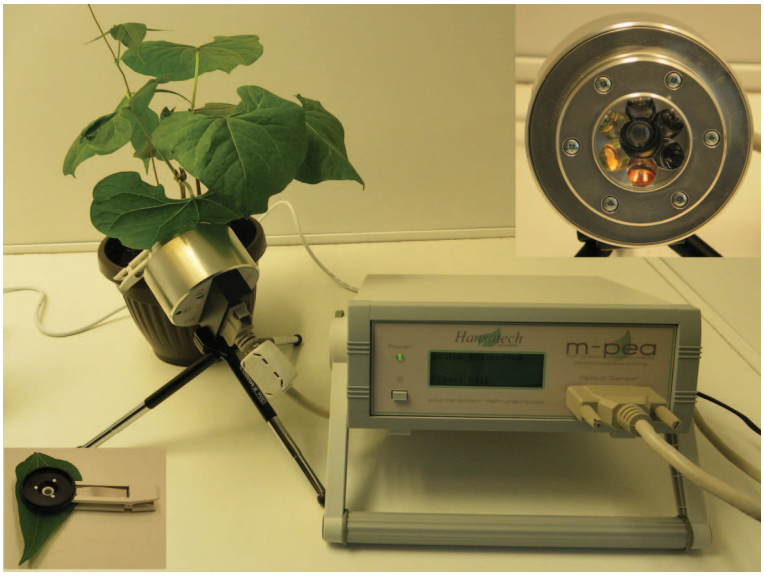

Fig. 8. Multi-Function Plant Efficiency Analyzer, M-PEA (of Hansatech, UK). It allows the measurement of several signals: prompt (Chl *a*) fluorescence, delayed (Chl *a*) fluorescence, P700 absorbance change, and relative chlorophyll content. The device separates the signals of prompt and delayed fluorescence electronically 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 a optical sensor unit (shown 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 is by one of the authors (VG)

The M-PEA simultaneously measures the PF and the modulated reflectance (MR) at 820 nm, related to P700 changes (Schansker et al., 2003); short dark intervals, ranging from μs to ms can interrupt the light phase of a PF transient, during which the DF kinetics are recorded with the same data acquisition system as PF and MR. The redox state of the PS II primary electron quinone acceptor (Q_A), which is reflected in the relative variable prompt fluorescence $V_t = (F_t - - F_O)/(F_M - F_O)$ is dependent on the recombination reactions which provoke the DF signals. The redox state of the primary quinone electron acceptor of PS II, Q_A, depends on the redox states of the electron transport chain carriers, which, in turn, are affected by the redox state of PS I RC (P700) which determines the 820 nm light reflection (MR) kinetics. Therefore, the collection and correlation of complementary information on three parts of the photosynthetic electron transport are made possible by simultaneous *in vivo* measurements of PF, DF and MR — PS II electron donor side, electron transport between PS II and PS I, and PS I 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 towards, or away from the leaves, on one side only (usually the upper leaf side). By measuring leaf discs, the lower side of the leaf is free and available for simultaneous measurement of oxygen gas exchange in the second to minute time range. An adapted Hansatech Clark type oxygen electrode may indeed provide this information (see Strasser (1974), Strasser and Sironval (1974); for recent data and a technical arrangement, see Gururani et al. (2012)).

Comparison of simultaneously measured PF and DF

As we already know, in both prompt and delayed Chl fluorescence, light quanta are emitted from the same population of PS II antenna Chl molecules. A comparison and an understanding of the similarity and the differences between the two signals is given by the opportunity to experimentally record both types of light emission from the same sample at almost the same time. This may also provide additional information about the state of the photosynthetic machinery. The PF transient, plotted on the same time scale, is often compared with the DF induction curve in order 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 millisecond (ms) DF is usually a complex mix of fast and slow kinetic components, which behave in different ways during the induction period. This causes a problem in the comparison of DF and the PF transient (Mar et al., 1975). The ms DF, with a lifetime of 2–3 ms, does not correlate with changes in PF. A correlation has been observed, however, for longer DF components (Clayton, 1969; Malkin and Barber, 1978).

A so-called *phase diagram* is a good way to visualize correlation between PF and DF (Malkin et al., 1994). DF within different dark decay intervals can be plotted in a 2D graph as a function of relative variable fluorescence V_t. Every DF point is an averaged value of the DF signal collected from one of the 3 dark time windows: 20 to 90 μs (Figure 9, left, *a*), 100–900 μs (Figure 9, middle, *b*) and 1–2.3 ms (Figure 9, right, *c*). DF values are plotted against corresponding values of prompt fluorescence, recorded just before the dark interval used for DF measurement. Each of the 3 «phase diagrams» can be divided into 2 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 Ganeva et al., 1988.) PF and DF change in the same way in the linear part of the curves: thus, it may be assumed that the main cause of these changes is related to fluorescence quantum yield (Lavorel, 1975; Goltsev et al., 2003). PF and DF deviate from linearity in the fast phase since the photosynthetic reactions determining PF and DF changes affect them in different ways. It is possible to separate four parts of the phase diagram characterized by a specific type of correlation between DF and the variable PF, V_t (see Figure 9): **(1)** at the beginning of the induction curve, PF and DF increase simultaneously (DF phase O–I_1, time interval $t = 0.3$–11 ms); **(2)** DF decreases as 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 a simultaneous decrease in both the signals within the time interval of 5–300 s (DF phases I_4–S). To exploit this method, further research into these complex relationships is needed.

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 several photons in each PS II. The second type of correlation during DF change from the I_1 to I_3 reflects the closure of PS II RCs, and the formation of $S_iZP680Q_A^-Q_B^{2-}$ states that may produce slow DF component, but not the fast μs decaying component. The third type of correlation occurs during the I_3 to I_4 DF induction phase, and, perhaps, it is the result of photo-induced thylakoid membrane energization, which affects PF and DF in different ways.

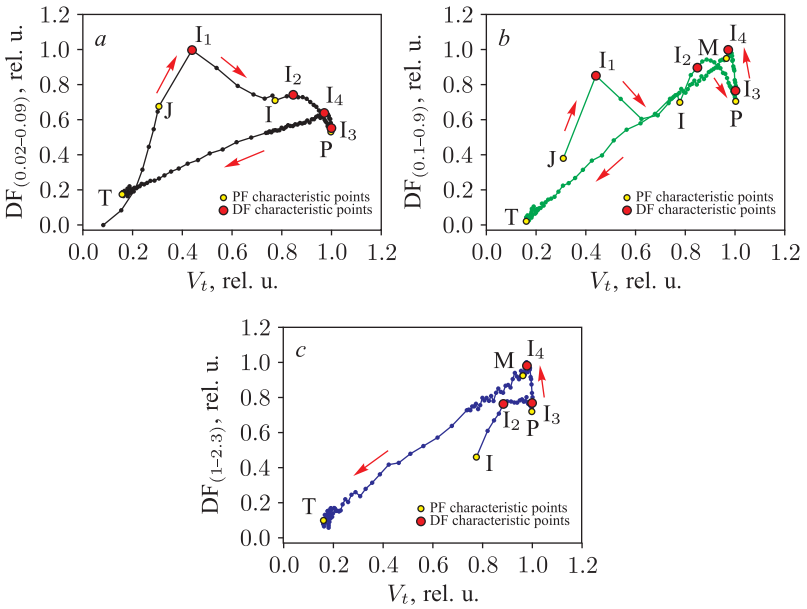

Fig. 9. Diagrams showing correlation between DF and relative variable PF, V_t. **Panel *a***: microsecond (20–90 μs) DF; **panel *b***: sub-millisecond (100–900 μs) DF; and **panel *c***: millisecond (1–2.3 ms) DF. Characteristic points of DF induction (labelled as $I_1 \ldots I_4$) are shown as solid (red) circles and of PF transient points (labelled as J, I, P, (S), M and T) as solid yellow circles (see text for details, and meaning of the symbols). This figure was drawn by one of the authors (VG), using his original data

Concluding remarks on the comparison of PF and DF

Light energy, absorbed by Chl *a* molecules of the antenna complexes, undergoes a series of successive transformations, before it is converted into chemical energy. Most light reactions of the photosynthetic process are principally reversible and the energy can be returned at any stage to its initial form and emitted as light quanta, although with low yields, as PF or DF. The re-emitted quanta of PF and DF contain important information about the forward and backward reactions which lead to the formation of the excited state of the antenna Chls.

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

$$^1P680 * Pheo \leftrightarrows P680^+Pheo^-. \quad (1)$$

Prompt fluorescence provides information on the structure and energy migration processes within photosynthetic antenna (Clegg et al., 2010). However, the redox equilibrium in the PS II reaction center is determined by subsequent redox reactions in the electron transport chain, and this enables the researcher using fluorescence to «see» different parts of the electron transport chain — from PS II acceptors, plastoquinone pool, and even the electron transfer from the PQ-pool to the PS I terminal acceptors (see Strasser et al., 2004, 2010; Tsimilli et al., 2008); cf. (Schreiber and Neubauer, 1987; Papageorgiou and Govindjee, 2011; Stirbet and Govindjee, 2011, 2012).

Regardless of the fact that the same population of antenna chlorophylls of PS II 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 a chemical redox reaction both on the donor and the acceptor side of PS II. Therefore, DF emission carries additional information not only on the concentration of the PS II redox states — the DF precursors, but about the rates of electron transport reactions in which they participate (Lavorel, 1975; Goltsev et al., 2005, 2009). Thus, the free energy differences between the excited-antenna state and the radical-pair state is given by DF (Grabolle and Dau, 2005); it is possible to calculate differences in energies of several PS II redox states. The redox-potentials of the electron carriers on the PS II donor side have been evaluated in several studies based on the measured DF decays (Grabolle and Dau, 2005; Zaharieva et al., 2011). The light-induced electron transfer and related processes can be quantitatively studied by using the DF emission of PS II as a tool (e. g., proton movement see Buchta et al., 2007; Dau and Zaharieva, 2009; and Zaharieva et al., 2011).

A new perspective has opened by simultaneous measurements, *in vitro*, *in vivo* or *in situ*, of PF and DF. This perspective uses these emissions as tools for photosynthesis research. 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 on the photosynthetic machinery, its structure and function.

Concluding Remarks

Prompt fluorescence has been greatly used in understanding excitation energy transfer from various photosynthetic pigments to Chl *a* (using the well-known «sensitised fluorescence» method). Using this method, already in 1922, G. Cario and James Franck excited a mixture of mercury (Hg) and thallium (Tl) vapour with light absorbed by Hg (254 nm), and observed emission spectra of both Hg and Tl. Since Tl was not excited, it was able to emit light (at 535 nm) only because of excitation energy transfer from Hg. This, then, was the first example of sensitised fluorescence (see Cario and Franck, 1922; Loria, 1925) for confirmation and extension of the concept). The doctoral thesis of Louis N. M. Duysens at the State University, Utrecht, The Netherlands (Duysens, 1952) used this method elegantly in photosynthesis research. Further, Govindjee et al. (1960) confirmed the existence of two-light reaction and two pigment systems through the observation of quenching of PS II fluorescence by PS I light, as implied in the discovery of the enhancement effect on oxygen evolution (Emerson et al., 1957). The key hypothesis that Chl *a* fluorescence intensity is inversely related to the concentration of Q_A, the first plastoquinone electron acceptor of PS II, was provided by Duysens and Sweers (1963) (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, CO_2 fixation and partial reactions of the entire photosynthetic chain can provide the full breadth of understanding of the phenomenon under investigation. There are now instruments available to measure photosynthesis and chlorophyll fluorescence in the same system (See e. g. CIRAS-3 at http://www.ppsystems.com/ciras3_portable_photosynthesis_system.htm and LI-COR's LI-6400XT at http://www.licor.com/env/applications/fluorescence.html.) However, since the future goals of obtaining biomass, biofuel and bioenergy depends on the efficiency of photosynthesis at all levels, the time has come to recommend to the major manufacturing companies the challenge of producing inexpensive instruments to simultaneously, and in parallel, measure fluorescence, whole chain electron flow, PS II and PS I activities, O_2 evolution and CO_2 uptake on algae, cyanobacteria and plant farms. **Appendix 1** provides a list of websites of some of the companies involved in manufacturing instruments related to the topic of this chapter.

The earliest observations by Sir G. G. Stokes (1819–1903) to our current status of using light emission as a tool in understanding the complex photosynthesis machinery has led us down

a winding and enlightening path. We end this historical and educational chapter by expressing our appreciation to David Walker (1928–2012), a master of communication of basic concepts of these processes to children as well as their elders.

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Appendix 1

Hansatech
http://www.hansatech-instruments.com/index.htm

LICOR
http://www.licor.com/env/applications/fluorescence.html

PPSystems
http://www.ppsystems.com/ciras3_portable_photosynthesis_system.htm

Photon Systems Instrumentation (PS I)
http://www.psi.cz/about-psi/our-company

WALZ
http://www.walz.com/

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Измерение *in vivo* светового излучения в растениях

Х. М. Каладжи, В. Гольцев, М. Брестич, К. Боса, С. И. Аллахвердиев, Р. Й. Страссер, Говинджи

Существуют несколько типов светового излучения у растений: быстрая флуоресценция, замедленная флуоресценция, термолюминесценция и фосфоресценция. Настоящая глава фокусируется на двух процессах: быстрой и замедленной флуоресценции. Измерение флуоресценции хлорофилла *a* проводили на протяжении более, чем 80 лет для изучения фотосинтеза, а начиная с 1961-го года, применяли в исследованиях 2-й фотосистемы (PS II).

В настоящее время методы, основанные на измерении флуоресценции, традиционно применяются в сельскохозяйственных и биологических исследованиях, в которых многие измеряемые и вычисляемые параметры используются в качестве биомаркеров и показателей устойчивости растений к различным абиотическим и биотическим стрессам. Такое стало возможным, благодаря быстрому усовершенствованию флуориметров. В основе работы этих приборов находятся два разных принципа измерения переменной флуоресценции хлорофилла *a*: 1) возбуждение, модулированное по амплитуде, с последующим измерением быстрой флуоресценции; 2) возбуждение сильным светом фотосинтетически активной радиации, приводящее к быстрой флуоресценции. Помимо флуориметров, были сконструированы приборы для измерения других сигналов, таких как замедленная флуоресценция, относящаяся, в основном, к PS II и изменение свето-индуцированного поглощения в результате фотоокисления реакционного центра PS I, P700, измеряемые, как уменьшение поглощения (фотовыцветание) при длине волны около 705 нм, или, по увеличению поглощения при длине волны 820 нм. Настоящая глава включает технические и теоретические принципы работы недавно появившихся приборов, которые позволяют одновременное измерение как быстрой (PF), так и замедленной (DF) флуоресценции вместе с некоторыми другими параметрами. Особое внимание уделяется сравнительному измерению PF и DF. DF менее известна, чем PF и реже используется, но, тем не менее, она также обладает большим потенциалом для получения полезной количественной информации об обратных реакциях переноса электронов в PS II. По этой причине, DF обсуждается более подробно.

Настоящая глава, хотя и описывает историю развития флуориметров, посвящается, также, Дэвиду Уокеру (1928–2012), который был пионером в области фотосинтеза и флуоресценции хлорофилла.