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TECHNICAL COMMUNICATION

New multichannel kinetic spectrophotometer–fluorimeter with pulsed measuring beam for photosynthesis research

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Abstract A multichannel kinetic spectrophotometer–fluorimeter with pulsed measuring beam and differential optics has been constructed for measurements of light-induced absorbance and fluorescence yield changes in isolated chlorophyll-proteins, thylakoids and intact cells including algae and photosynthetic bacteria. The measuring beam, provided by a short (2 μs) pulse from a xenon flash lamp, is divided into a sample and reference channel by a broad band beam splitter. The spectrum in each channel is analyzed separately by a photodiode array. The use of flash measuring beam and differential detection yields high signal-to-noise ratio (noise level of 2×10^{-4} in absorbance units per single flash) with negligible actinic effect. The instrument covers a spectral range between 300 and 1050 nm with a spectral resolution of 2.1, 6.4 or 12.8 nm dependent on the type of grating used. The optical design of the instrument enables measuring of the difference spectra during an actinic irradiation of samples with continuous light and/or saturation flashes. The time resolution of the spectrophotometer is limited by the length of Xe flash lamp pulses to 2 μs.

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Abbreviations

CCD	Charge coupled device
Chl	Chlorophyll
D1	*psbA* gene product
LED	Light emitting diode
PDA	Photodiode array
Pheo	Pheophytin
PS II	Photosystem II
RC	Reaction centre
T–S	Triplet-minus-singlet
UV–VIS–NIR	Ultraviolet–visible–near infrared

Introduction

Kinetic UV–VIS–NIR absorption spectroscopy is a powerful tool that is often used in photosynthesis research. This method has played crucial role in revealing the nature and kinetics of electron transfer in various photosynthetic pigment–protein complexes, identifying electron carriers, following the dissipation of excitation energy through triplet states and many other processes (for review see Diner and Rappaport 2002). Such measurements require instrumentation with high sensitivity and fast response matching the time scale of the reactions, because only a small fraction of molecules contributing to the overall absorbance is usually involved in photochemical reactions. Devices capable of measuring absorbance (A) changes as small as 0.001 on millisecond,

microsecond or even shorter time scales are often necessary. Usually, these absorbance changes are measured using single wavelength spectrophotometers. These techniques include a pumping light source (flash lamp or laser), very stable weak measuring light source (tungsten lamp or laser) covering the required spectral range and a fast detector (Hillmann et al. 1995). Optionally, the measuring light is provided by a pulsed light source with its intensity variations being corrected by a reference. Previously, Joliot et al. (1980) and later Kramer and Crofts (1990) and Kramer and Sacksteder (1998) designed instruments with pulsed measuring light for measurements of small absorbance changes in blue–green spectral region. Several other groups also utilized pulsed measuring light in their instruments (Klughammer and Schreiber 1994; Bukhov and Carpentier 2003; Naqvi et al. 2003).

Single wavelength instruments meet the demands of low exposure of the samples and fast response time. On the other hand, single wavelength measurement does not allow measurements of interfering signals, where deconvolution using several spectra points is needed to extract contributions from individual components. Measuring light-induced difference spectra ''wavelength by wavelength'' is time consuming, requires high stability of the sample during the measurement, and, especially, prevents recording of spectra of unrepeatable transients.

Multichannel detection is frequently used for measurement of absorption in transparent samples see e.g. Janata (2003). The major disadvantage of multichannel measurement is that high measuring intensities have to be used to give sufficient signal on all channels. Though the mean measuring intensity can be reduced by use of light pulses triggered with the measurement (Sacksteder et al. 2001), low reproducibility of pulses limits the precision of such measurements and the signal often has to be averaged over hundreds to thousands traces. Kinetic spectral measurements can also be performed with a single channel instrument in which different points of a spectrum are measured sequentially by applying a series of LED flashes, each filtered by an interference filter to provide different wavelength probes (Klughammer et al. 1990). This approach was developed to measure small absorbance changes on scattering samples.

In this contribution, we present a newly developed multichannel spectrophotometer–fluorimeter with pulsed measuring light for measurements of light-induced absorbance changes in both transparent and scattering samples. Depending on application, various multichannel detection units can be used to read out the spectrum during one 2-μs long flash with noise level of 2×10^{-4}. Moreover, simultaneous measurement of fluorescence intensity emitted from the sample is possible.

Experimental apparatus

Our new multichannel kinetic spectrophotometer–fluorimeter is based on a pump-probe method, which is known from laser-based techniques. A sample is at first exposed to a strong flash causing photochemical reaction in the sample. Then, with a time delay, the second weak measuring flash is applied to analyze the photochemical change. In our instrument, instead of monochromatic laser pulses, we use broadband xenon flashes as measuring light pulses. Such arc lamp flashes suffer from low reproducibility of light intensity and spectral distribution. Therefore, to get a better precision, a correction for the flash intensity throughout the used spectral range is necessary. This is accomplished by splitting the measuring beam into two pathways. One is used for sample measurement and another for intensity and spectral correction. Both beams are dispersed in single imaging monochromator and the two spectra are detected by multichannel detectors such as photodiode arrays (PDA) or charge coupled devices (CCD).

The scheme of the spectrophotometer is presented in Fig. 1. The instrument is composed of a measuring flash lamp (FL1), actinic light sources (FL2, AL), optical system (C, O1–O3, BS, M1–M3), monochromator (MONO), detector (PDA) and a control unit. The measuring light is provided by a short arc xenon flash lamp (FL1) (FX 1161, EG&G, USA) giving flashes with pulse width of 2 μs. Energy of measuring flashes can be regulated by adjusting the voltage and by the choice of the discharge capacitor, up to 0.5 J per pulse. The measuring light is focused by a quartz condensor (C) to a quartz glass rod (O1, 1.5 mm

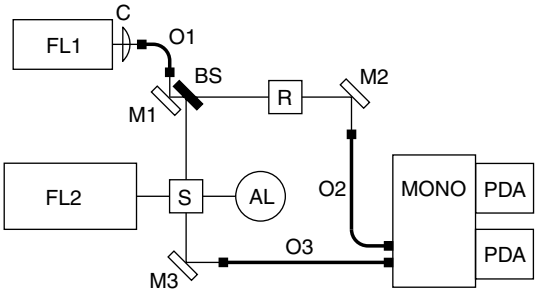

Fig. 1 Scheme of the instrument. See the main text for details. FL1, FL2: Xe flash lamps; M1, M2, M3: toriodal mirrors; O1: quartz rod, O2, O3: fiber optic bundles; C: condensor; BS: beam splitter; S, R: sample and reference cuvette holders; AL: actinic lamp; MONO: monochromator MS 247; PDA: photodiode arrays

diameter, 20 cm length), which homogenates the light intensity across the rod area, and then collimated to the samples by a toroid mirror (M1). The light beam, expanded by the collimator to the area of 0.8 cm^2, is divided into the sample and reference channels by a broadband beam splitter (BS). After passing through the sample and reference, both light beams are focused, again by toroid mirrors (M2, M3), into two independent fibre optic bundles delivering the light to a flat field imaging monochromator (MONO) (MS 257, Oriel, USA). The monochromator is equipped with three gratings giving it the spectral resolutions of 2.1, 6.4 and 12.8 nm per mm. Optical parts were manufactured by Optical Development Workshop of the Academy of Sciences of the Czech Republic, Turnov.

Each absorbance detector unit is build up from two photodiode arrays composed of either (i) Si photodiode linear array with 38 large area (0.9×4.5 mm) elements (PDA, S4111, Hamamatsu, Japan), and two amplifiers (C2334, Hamamatsu, Japan) or (ii) thermoelectrically cooled NMOS linear image sensor with 512 elements (NMOS C5964, Hamamatsu, Japan). The actinic light is provided by a high power (4 mJ of discharge energy per pulse) xenon flash lamp (FL2) (FX-249, EG&G, USA), a flash light emitting diodes unit (Photon System Instruments, Brno, Czech Republic) or by continuous light from a halogen lamp source coupled to fibre bundle (AL) (KL 1500, Walz, Germany). The light sources can be mounted to the main body of the instrument simultaneously and combined in different experiments.

The signal and reference data are collected and processed by a control unit FL 200 (Photon System Instruments, Brno, Czech Republic). This unit is used for driving PDA amplifiers and triggering and measuring actinic light sources. The control unit allows triggering with the precision of 100 ns and repetition rate of 500 ns. Both PDAs are read out simultaneously. The minimal read out time is 1.5 ms. Measuring sequences are under complete control of the user and case-to-case individualization of measuring procedures including precise timing of flashes, PDA read-out and fluorescence measurement is possible via control unit software.

The optical system acts as a double collimator. The first collimator expands the light beam and directs it to the sample. The second one focuses the light to an optical fibre attached to a monochromator. The expansion of the measuring beam diameter from 1.5 mm (xenon arc size) to about 9 mm at the sample plane reduces the intensity of measuring light per unit area of the sample by a factor of 36. This is important for keeping the measuring light intensity low enough to minimize its actinic effect. Focusing the measuring beam back to 1.5 mm fibre optics allows the use of small area detectors, which are components of such multichannel detectors as PDAs or CCDs.

Using small area of the light input into the monochromator (fibre optics with core diameter of 1.5 mm) reduces scattered light and fluorescence by a factor of 10^4 and thus effectively prevents them from disturbing the absorption measurement. This allows absorbance measurement of highly fluorescent samples and acquiring the spectra during actinic light illumination without any filtration of actinic light by blocking filters. Since the time resolution of the instrument is determined by the width of the measuring pulse (2 μs) the instrument is capable of capturing events occurring even on time scales shorter than the width of actinic pulse (2 μs). Nevertheless, the level of diffused light rejection is not sufficient for some experiments requiring high intensity of actinic exposure as, for instance, triplet-minus-singlet spectra measurement or measurements with highly scattering samples. In these cases, diffused light should be removed from the spectrum by subtracting the signal taken without the measuring flash.

The maximal repetition rate is limited by charging of the Xe lamp discharge capacitor. It depends on the rate of charging, i.e., on the power supply, and on the capacitance of the capacitor. For our instrument, it ranges from 35 Hz (20 W power supply, 1 μF capacitor) to 300 Hz (60 W power supply, 0.2 μF capacitor). The choice of the capacitor depends on the light intensity required to give a sufficient signal to the detector. The measuring light intensity reaching the detector depends on the required spectral resolution, light scattering and optical density of the sample. Measurements presented in this contribution were performed with 20 W power supply and 0.5 or 1 μF capacitor.

The instrument control unit, FL200, is primarily designated for measurements of chlorophyll fluorescence yield. Therefore, its native PIN diode detector can also be used for measurements of fluorescence kinetics simultaneously with absorbance. In our instrument, the PIN diode mounting point is above the sample cuvette, in the direction perpendicular to both measuring and actinic light beams. The spectral region in which the fluorescence is detected can be selected by an appropriate filter. The instrument allows measurements of the fluorescence yield using a modulated measuring light provided by a set of light-emitting diodes. Duration, intensity and timing of measuring flashes and recording of the fluorescence signal are controlled independently of the measurements of absorbance, which offers a high flexibility to the design of experiments.

Results and discussion

Difference spectra

Figure 2 shows three examples of room temperature single flash-induced absorbance difference spectra of oxidized chlorophyll (Chl^+), reduced pheophytin ($Pheo^-$) and triplet minus singlet spectrum (T–S) in isolated reaction centres of photosystem II (PSII). (i) Chlorophyll cation can be accumulated in PSII reaction centres (RC) on D1 accessory chlorophyll molecule in the presence of artificial electron acceptor silicomolybdate (Vácha et al. 2005). The spectrum has characteristic features reported previously (Barber et al. 1987; Telfer et al. 1990; Vácha et al. 2002). (ii) $Pheo^-$ can be accumulated in PSII RC in the presence of artificial electron donor. The spectrum reported here resembles those reported previously (Nanba and Satoh 1987; Barber et al. 1987; Vácha et al. 2002). (iii) T–S spectra can be measured in PSII RC under conditions when chlorophyll molecules convert to their triplet states by a radical pair recombination mechanism (Okamura et al. 1987). In all reports, so far, only Q_y part of the chlorophyll spectrum (Van Kan et al. 1990; Kwa et al. 1994; Hillmann et al. 1995; Eijckelhoff et al. 1997) is presented. Here, the T–S spectrum of PSII RC covers whole visible spectral region.

Chlorophyll triplet relaxation and T–S spectroscopy

Figure 3 shows a flash-induced absorbance changes corresponding to kinetics of triplet states relaxation at 682 nm at room temperature in the isolated PSII RC in the presence of oxygen. The original difference spectra taken in time range of 3–350 μs are inserted. The relaxation is a single exponential process with time constant of 44.4 ± 0.9 μs, which is in a good accordance with the value of 33 ± 3 μs reported by Durant et al. (1990). Signal-to-noise ratio was better than 200/1, the intensity of the actinic Xe flash lamps was proven to be sufficient to induce triplet-minus-singlet spectra of chlorophyll *a* in organic solvents (results not shown).

Another example of the threshold resolution measurement is in Fig. 4 showing spectra corresponding to a triplet state decay of carotenoid spheroidenone in RC of purple bacterium *Rhodobacter sphaeroides*, antenna deficient strain RCO2 (McGlynn et al 1994). Each spectrum represents a single flash measurement without averaging. Kinetics of ΔA600–ΔA705 was single exponential with the lifetime of 8.4 ± 0.4 μs. Comparable results on the reaction centres of wild type and mutant *R. sphaeroides* were obtained by Arellano (Arellano et al. 2004).

Time course measurements

An example of a simultaneous measurement of absorbance changes and fluorescence yield during a continuous actinic illumination on whole cells of the purple bacterium *R. sphaeroides* is given in Fig. 5. The kinetic traces correspond to a fluorescence induction kinetics (solid line), bacteriochlorophyll oxidation (open circles) measured as an absorbance change at 602 nm (ΔA_{602}) and an electrochromic shift (open triangles) of carotenoid absorption bands plotted as a

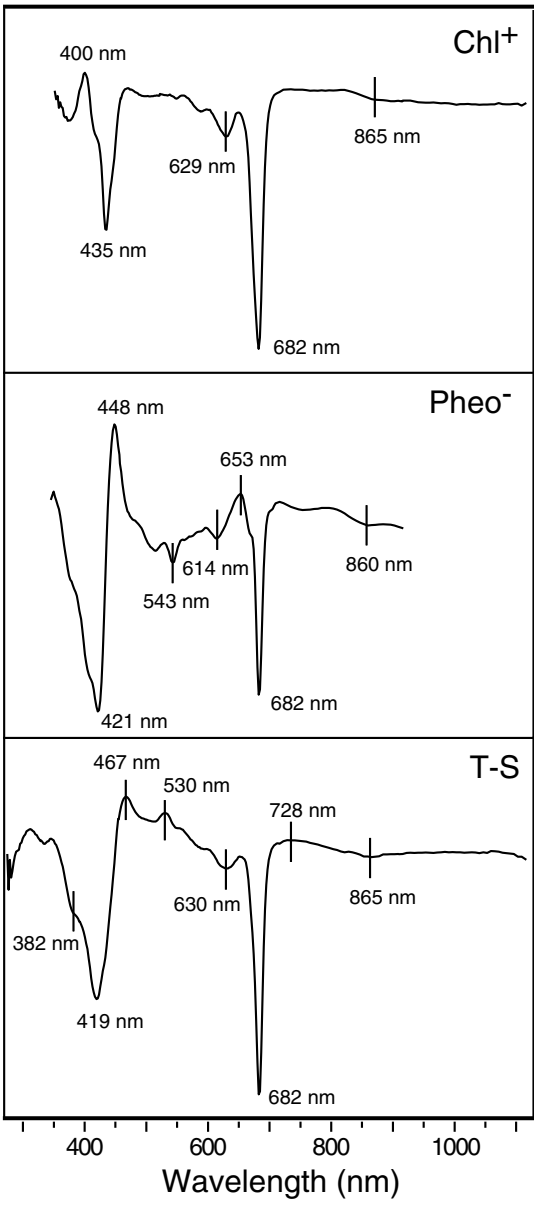

Fig. 2 Room temperature single flash detected absorbance difference spectra of oxidized chlorophyll (Chl^+), reduced pheophytin ($Pheo^-$) and triplet minus singlet spectrum (T–S) in isolated reaction centres of photosystem II (PSII)

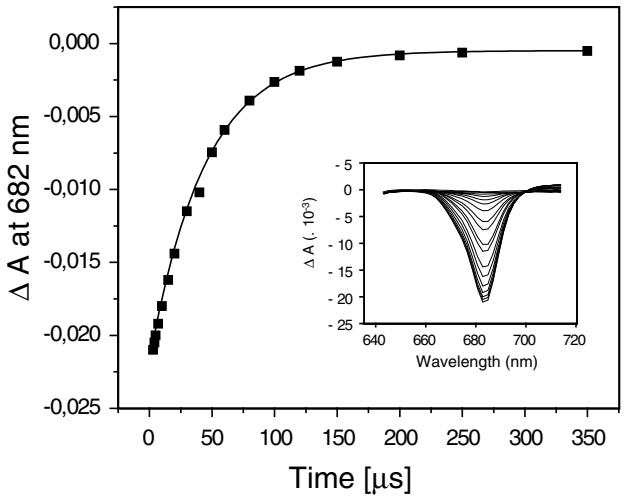

Fig. 3 Chlorophyll triplet states relaxation in the isolated reaction center of photosystem II in the presence of oxygen at room temperature (total chlorophyll concentration in the sample was 10 μg/ml). The relaxation has single exponential decay with time constant 44.4 μs. Data were obtained by a pump-probe method using xenon flash lamps for actinic and measuring pulses. Inserted: individual T–S spectra of PSII RC at different time delays during the triplet relaxation

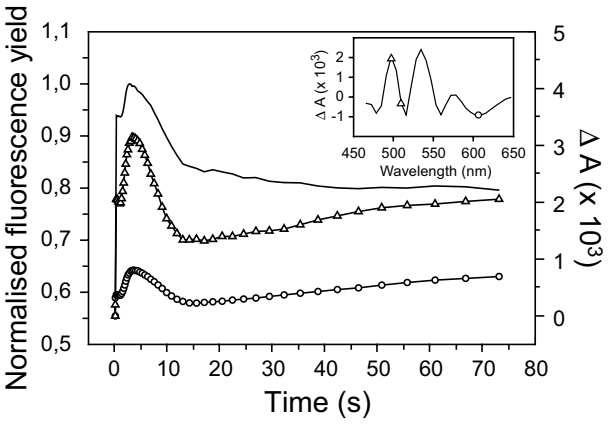

Fig. 5 Kinetics of the fluorescence yield (solid line), light-induced absorbance changes corresponding to bacteriochlorophyll oxidation (open circles) and an electrochromic shift of carotenoid absorption (open triangles) measured simultaneously on a suspension of whole cells of the purple bacterium *Rhodobacter sphaeroides*. Bacteriochlorophyll oxidation is plotted as the absorbance change at 602 nm (ΔA_{602}), electrochromic shift is plotted as the difference in absorbance change at 490 and 510 nm (ΔA_{490}–ΔA_{510}). Traces represent an average of five measurements. Inserted: the example of a light-induced difference spectrum, from which the kinetics were computed. The kinetics of absorbance changes were calculated from positions marked in the spectrum by open circle for bacteriochlorophyll oxidation and open triangles for electrochromic shift

difference in absorbance change at 490 and 510 nm (ΔA_{490}–ΔA_{510}). The electrochromic shift serves as a measure of an electric potential built up on the photosynthetic membrane (De Grooth and Amesz 1977). The inset in Fig. 5 presents an example of a light-induced difference spectrum. The actinic irradiance of 2000 μmol m^{-2} s^{-1} was provided by a halogen lamp KL 1500 (Walz, Germany).

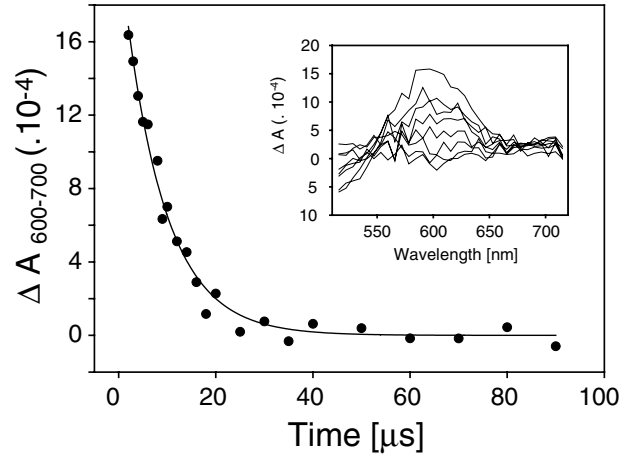

Fig. 4 Carotenoid triplet state relaxation in whole cells of photosynthetic purple bacterium *Rhodobacter sphaeroides* measured at room temperature. The decay was fitted with single exponential with time constant 8.4 μs. Inserted: the individual carotenoid triplet–triplet absorption spectra measured by single flash during the course of the triplet relaxation

Conclusions

Multichannel kinetic absorption spectrometer with an option for measurement of fluorescence presented here is a useful and easy tool for measurement of absorption kinetics in microsecond time-scales. The advantages of the multichannel spectrophotometer with dual detection configuration combined with a single imaging spectrograph are mainly the single flash measurement mode and high signal to noise ratio. For microsecond kinetics, the Xe flash lamp is a relatively cheap alternative compared to a laser operated pump and probe kinetic setups. For more details about the research carried out using this instrument see Bertrand et al. (2001), Vácha et al. (2002), Zehetner et al. (2002), Vácha et al. (2003), Litvin et al. (2005) and Vácha et al. (2005).

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References

Arellano JB, Melo TB, Cogdell RJ, Fyfe PK, Naqvi KR (2004) Multi-channel flash spectroscopy of the reaction centers of wild type and mutant *Rhodobacter sphaeroides*: bacteriochlorophyll B-mediated interaction between the carotenoid triplet and the special pair. Photochem Photobiol 79:68–75

Barber J, Chapman DJ, Telfer A (1987) Characterisation of a PS II reaction centre isolated from the chloroplasts of *Pisum sativum*. FEBS Lett 220:67–73

Bertrand M, Schoefs B, Šiffel P, Roháček K, Molnar I (2001) Cadmium inhibits epoxidation of diatoxanthin to diadinoxanthin in the xantophyll cycle of the marine diatom *Phaeodactylum triornutum*. FEBS Lett 508:153–156

Bukhov NG, Carpentier R (2003) Measurement of photochemical quenching of absorbed quanta in photosystem I of intact leaves using simultaneous measurements of absorbance changes at 830 nm and thermal dissipation. Planta 216:630–638

De Grooth BG, Amesz J (1977) Electrochromic absorbance changes of photosynthetic pigments in *Rhodopseudomonas sphaeroides*. I. Stimulation by secondary electron transport at low temperature. Biochim Biophys Acta 462:237–246

Diner BA, Rappaport F (2002) Structure, dynamics and energetics of the primary photochemistry of Photosystem II of oxygenic photosynthesis. Annu Rev Plant Biol 53:551–580

Durrant JR, Giorgi LB, Barber J, Klug DR, Porter G (1990) Characterisation of triplet states in isolated Photosystem II reaction centres: oxygen quenching as a mechanism for photodamage. Biochim Biophys Acta 1017:167–175

Eijckelhof C, Vacha F, van Grondelle R, Dekker JP, Barber J (1997) Spectroscopic characterization of a 5 Chl *a* Photosystem II reaction center complex. Biochim Biophys Acta 1318:266–274

Hillmann B, Brettel K, van Mieghem F, Kamlowski A, Rutherford AW, Schlodder E (1995) Charge recombination reactions in photosystem II. 2 Transient absorbency difference spectra and their temperature-dependence. Biochemistry 34:4814–4827

Janata E (2003) Instrumentation of kinetic spectroscopy.17. The use of a diode array multichannel detector in kinetic experiments. Rad Phys Chem 66:145–148

Joliot P, Beal D, Frilley B (1980) A new spectrophotometric method for the study of photosynthetic reactions. J Chem Phys 77:209–216

Klughammer C, Kolbowski J, Schreiber U (1990) Led array spectrophotometer for measurement of time resolved difference spectra in the 530–600 nm wavelength region. Photosynth Res 25:317–327

Klughammer C, Schreiber U (1994) An improved method, using saturating light-pulses, for the determination of photosystem I quantum yield via $P700^{+}$ absorbency changes at 830 nm. Planta 192:261–268

Kramer DM, Crofts AR (1990) Demonstration of a highly-sensitive portable double-flash kinetic spectrophotometer for measurement of electron-transfer reactions in intact plants. Photosynth Res 23:231–240

Kramer DM, Sacksteder CA (1998) A diffused-optics flash kinetic spectrophotometer (DOFS) for measurements of absorbance changes in intact plants in the steady-state. Photosynth Res 56:103–112

Kwa SLS, Eijckelhoff C, Van Grondelle R, Dekker JP (1994) Site-selection spectroscopy of the reaction center complex of photosystem II.1. Triplet-minus-singlet absorption difference—search for a 2nd exciton band of P680. J Phys Chem 98:7702–7711

Litvin R, Bina D, Siffel P, Vacha F (2005) Conformational changes and their role in non-radiative energy dissipation in photosystem II reaction centres. Photochem Photobiol Sci 4:999–1002

McGlynn P, Hunter CN, Jones MR (1994) The *Rhodobacter sphaeroides* PufX protein is not required for photosynthetic competence in the absence of a light harvesting system. FEBS Lett 349:349–353

Nanba O., Satoh K (1987) Isolation of a Photosystem II reaction center containing D-1 and D-2 polypeptides and cytochrome b-559. Proc Natl Acad Sci USA 84:109–112

Naqvi KR, Melo TB, Sliwka HR, Mohamad SBB, Partali V (2003) Photochemical and photophysical behaviour of vitamin E: interaction of its long-lived transient photoproducts with carotenoids. Photochem Photobiol Sci 2:381–385

Okamura MY, Satoh K, Isaacson RA, Feher G (1987) Evidence of the primary charge separation in the D1D2 complex of photosystem II from spinach: EPR of the triplet state. In: Biggins J (ed) Progress in photosynthesis research, vol. 1. Martinus Nijhoff, Dordrecht, pp 379–381

Sacksteder CA, Jacoby ME, Kramer DM (2001) A portable, non-focusing optics spectrophotometer (NoFOSpec) for measurements of steady-state absorbance changes in intact plants. Photosynth Res 70:231–240

Telfer A, He W-Z, Barber J (1990) Spectral resolution of more than one chlorophyll electron donor in the isolated Photosystem II reaction center complex. Biochim Biophys Acta 1017:143–151

Vácha F, Durchan M, Šiffel P (2002) Excitonic interactions in the reaction centre of photosystem II studied by using circular dichroism. Biochim Biophys Acta 1554:147–152

Vácha F, Kutý M, Durchan M, Šiffel P, Pšenčík J (2003) Localisation of accumulated chlorophyll cation in reaction centre of photosystem II. Mater Struct 10:21–24

Vácha F, Pšenčík P, Kutý M, Durchan M, Šiffel P (2005) Evidence for localisation of accumulated chlorophyll cation on the D1-accessory chlorophyll in reaction centre of photosystem II. Photosynth Res 83:279–302

Van Kan PJM, Otte SCM, Kleinherenbrink FAM, Nieveen MC, Aartsma TJ, Van Gorkom HJ (1990) Time-resolved spectroscopy at 10 K of the Photosystem II reaction center; deconvolution of the red absorption band. Biochim Biophys Acta 1020:146–152

Zehetner A, Scheer H, Siffel P, Vacha F (2002) Photosystem II Reaction Centre with altered pigment-composition: reconstitution of a complex containing five chlorophyll *a* per two pheophytin *a* with modified chlorophylls. Biochim Biophys Acta 1556:21–28