

# High resolution emission spectra of one second delayed fluorescence from chloroplasts

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The first well resolved emission spectra of white light-illuminated spinach chloroplasts at room temperature show that one second delayed fluorescence occurs at 685 nm. We demonstrate that reabsorption of this delayed fluorescence induces the second (probably prompt) emission observed at 730 nm and which we identify with the photosystem I peripheral antenna system.

Delayed fluorescence; Chloroplast; Emission spectrum; Photosystem II; Photosystem I; Reabsorption

## 1. INTRODUCTION

Absorption of light in higher plants and in isolated chloroplasts leads to two qualitatively different kinds of fluorescence: prompt fluorescence of excited chlorophyll molecules decays within nanoseconds while light-induced chemical reactions can result in delayed fluorescence which decays with characteristic times ranging from microseconds to minutes or longer. Delayed fluorescence thus provides a means of probing photosynthetic electron transfer processes. As early as 1954, when current knowledge of the photosynthetic apparatus was not available, physical arguments were advanced that prompt and delayed fluorescence spectra should be similar, the latter representing reexcitation of chlorophyll by reversed photosynthetic reactions [1]. Studies of delayed fluorescence, however, have been largely limited to measurement of the decay kinetics.

From studies of the kinetics of fluorescence delayed into the seconds regime (for review see [2]),

it is thought that delayed fluorescence is a result of recombination between positive and negative charges stored on the donor and acceptor sides, respectively, of photosystem II [3-5]. The actual emitters of delayed fluorescence are predicted to be chlorophyll molecules [1] located in photosystem II. To investigate this problem in more detail, well resolved delayed fluorescence spectra are required.

While the spectral composition of prompt fluorescence has been extensively investigated at cryogenic or physiological temperatures, there have been few studies of microsecond delayed fluorescence spectra [6] and almost none (except for the early pioneering work of Arnold and Davidson [1]) at longer delay times. This situation has been due largely to the inability to detect this extremely weak emission; even microseconds after excitation, the intensity of delayed fluorescence is some three orders of magnitude lower than that of prompt emission. The use of narrow band filters leads to further reduction of the light available at the detector while broad band filters do not provide well resolved spectra.

To overcome this difficulty, we have developed a method of ultraweak spectroscopy, utilizing two-dimensional single photon counting, which makes

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routinely accessible high resolution spectra of delayed fluorescence on the scale of seconds or longer from isolated chloroplast samples with concentrations as low as 1  $\mu\text{g}/\text{ml}$  [7]. Delayed fluorescence spectroscopy is suitable for physiologically realistic conditions such as isolated chloroplast preparations at room temperature or even leaves. Here we report high resolution measurements of long delayed fluorescence spectra and their concentration dependence, and discuss the origins of the observed emissions.

## 2. MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as described previously [8] and suspended in a Hepes buffer (50 mM Hepes, pH 7.2, containing 0.4 M sorbitol, 10 mM NaCl, 5 mM  $\text{MgCl}_2$ , 2 mM EDTA and 1 mM  $\text{MnCl}_2$ ) to approximately 1500  $\mu\text{g}/\text{ml}$  chlorophyll concentration. This suspension was diluted further with the same buffer to the concentrations required. Chlorophyll concentration (chlorophyll-*a* plus chlorophyll-*b*) was determined using an absorption spectroscopy method detailed in [9].

An imaging photomultiplier tube, cooled housing and associated data acquisition system (PIAS, Hamamatsu Photonics, Hamamatsu, Japan) were used to obtain the spectra reported here. The imaging tube has a multi-alkali photocathode with a diameter of 15 mm and useful response over the spectral range of approximately 300 nm to 850 nm. The tube is cooled to approximately  $-20^\circ\text{C}$  and, with the discriminator adjusted to optimize the signal-to-noise ratio, the dark noise count rate is about 1 count per second over a display consisting of  $512 \times 512$  pixels.

Spectra of delayed fluorescence were obtained by detecting the first order diffracted image (transparent grating; 400 lines/mm; Milton Roy, USA) of a slit (Melles Griot) mounted on a flow cell through which the samples were pumped. Using a 100  $\mu\text{m}$  slit, 1–1.5 nm resolution is obtainable. Calibration spectra were obtained by use of a monochromator and verified by comparison with a mercury-xenon lamp. The dispersion of the instrument is 1.1 nm/display pixel. A more detailed account of the detector and the spectroscope are to be found in [10,11].

Dark adapted, isolated chloroplasts were pumped to the flow cell (Masterflex peristaltic pump, Cole Parmer Instrumentation Co., USA) from a reservoir via black Viton tubing. Samples were illuminated for 3 s while passing through a segment of glass tube inserted into the pumping system. Isolation of the spectroscope from the excitation tube enabled us to use white light excitation (spectral range approximately 400–750 nm) provided by a microscope lamp (Olympus, Japan). Saturating effect of the illumination can be shown by the failure of the signal to increase with an increase in the excitation light intensity. The pump speed was adjusted so that the chloroplast suspension reached the flow cell 1 s after illumination. Photon counting data were transferred to a personal computer (NEC, Japan) and were corrected for noise and for the wavelength response of the photomultiplier tube (data provided by Hamamatsu Photonics).

As with any single photon counting measurement, the effects of fluctuations (arising from the quantum detection process and source fluctuations) are evident in the spectra presented here. The relative magnitude of the fluctuations is diminished by acquiring more photon counts, i.e., by extending the time of measurement. Spectra reported here were measured in the minimum time required to obtain clearly discernible spectral features.

## 3. RESULTS AND DISCUSSION

Wavelength dependence of the intensity of delayed fluorescence measured one second after white light excitation of a sample containing 10  $\mu\text{g}/\text{ml}$  of chlorophyll is shown in fig. 1a. The spectrum consists of a main peak at 685 nm and a lower, broader peak near 730 nm. The simplicity of the room temperature emission spectrum of functioning chloroplasts is interesting given the wealth of reported prompt fluorescence peaks.

Previous studies of prompt fluorescence may serve as a guide in interpreting our delayed fluorescence spectra. At 77 K prompt fluorescence is known to be composed of three emission bands peaking at 680 nm, 685 nm and 695 nm, and of a broad composite band between 710 and 750 nm [12]. The relative amplitudes of these peaks are temperature dependent; at room temperature the prompt fluorescence spectrum peaks at 685 nm with only a small contribution of the longer wavelength emission [13,14]. The three peaks below 700 nm are generally assigned to photosystem II while the longer wavelength bands are known to be connected with photosystem I [12–15]. Recent low temperature studies suggest that the 680 nm peak belongs to the light harvesting protein complex [16], the 685 nm peak to the photosystem II core complex with a minor contribution from the light harvesting protein complex [17,18], while the 695 nm emission belongs to the photosystem II core complex and partially, if at all, to photosystem I [19,20]. The long wavelength emission between 710 nm and 750 nm has previously been attributed to photosystem I [12–15,21,22], although the assignments of individual peaks to specific photosystem I functional units have been less clearly established than are those of photosystem II, and a possible contribution from photosystem II has not been excluded [16].

Our observation of a 685 nm delayed fluorescence peak provides direct experimental evi-

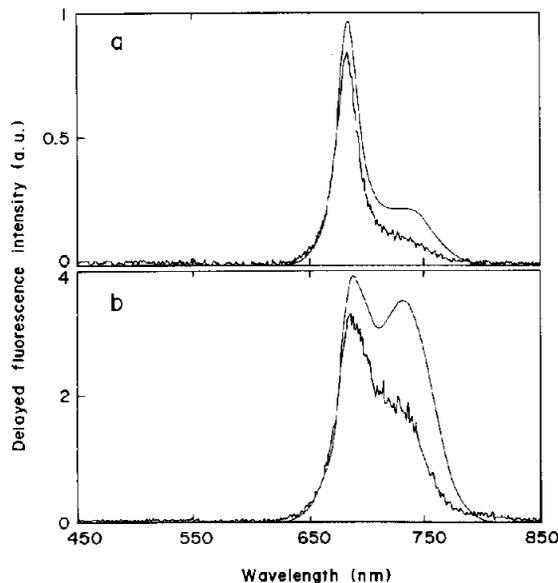


Fig. 1. Delayed fluorescence emission spectra of isolated spinach chloroplasts 1 s after white light excitation at room temperature. Chlorophyll concentrations are (a) 10  $\mu\text{g/ml}$  and (b) 100  $\mu\text{g/ml}$ . The data are comprised of  $10^5$  photon counts acquired in approx. 3.5 h. The smooth curve is the spectrum corrected for wavelength response of the detector (scale reduced by a factor of 22 for ease of comparison).

dence supporting the hypothesis which explains delayed fluorescence in terms of charge recombination resulting in excitation of photosystem II chlorophyll [23] and we conclude further that it is a core complex chlorophyll which is the site of delayed fluorescence emission. This conclusion is supported by a recent study of the prompt fluorescence spectra of purified photosystem II reaction centers which peak at 685 nm both at low [24,25] and at room temperature [26]. A known prompt fluorescence peak at the same wavelength as the delayed fluorescence maximum in our data not only strengthens previous assertions that delayed fluorescence decaying in seconds originates from photosystem II but suggests the same emission site as well.

In addition to the mean peak at 685 nm we observe a single, although broad, emission feature near 730 nm one second after excitation which becomes more prominent at higher concentrations (fig. 1b). To investigate the nature of this emission peak we have measured the concentration dependence of the one second delayed fluorescence

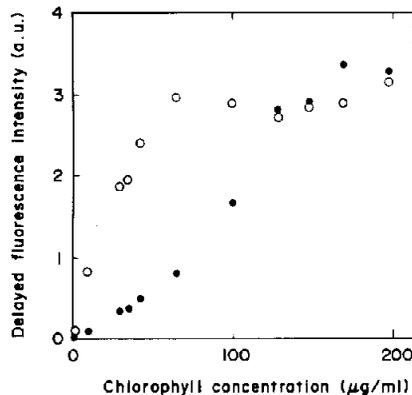


Fig. 2. Concentration dependence of 685 nm ( $\circ$ ) and 730 nm ( $\bullet$ ) delayed fluorescence emission intensities in isolated spinach chloroplasts 1 s after white light excitation.

spectrum over the chlorophyll concentration range of 1 to 200  $\mu\text{g/ml}$ . Fig. 2 shows the intensities of the 685 and 730 nm peaks (open and full circles, respectively) as a function of concentration.

The two emission intensities exhibit markedly different concentration dependencies. This signifies that the processes responsible for the two emissions differ fundamentally. The behavior of the intensities at low concentrations is of interest here. In the presence of absorption by the sample the intensity observed at the detector (fig. 2) is expected to be linear in concentration, in the low concentration regime. Within the accuracy of our data, the 685 nm intensity is linear at low concentrations, as seen in this figure.

The 730 nm intensity, however, clearly exhibits a quadratic concentration dependence, for low concentrations, which is the signature of a reabsorption-induced emission process. This follows from consideration of the Beer-Lambert law. In such a process the induced intensity is proportional to both the concentration and the intensity of the inducing light, in this case the delayed 685 nm emission. The latter is also proportional to concentration, leading to a quadratic dependence. The data in fig. 2 establish that in a chloroplast preparation, 685 nm delayed fluorescence is reabsorbed giving rise to a secondary emission at 730 nm. Within the accuracy of our data, the low concentration behavior of the 730 nm emission intensity is simply quadratic; the absence of a linear component suggests that the reabsorption induced emission is a prompt rather than delayed fluorescence.

The possibility of non-radiative energy transfer between excited photosystem II core complex chlorophylls (the suggested 685 nm emitters) and the species responsible for the 730 nm emission seems unlikely. The strong concentration dependence of the process indicates that it occurs between individual chloroplasts rather than in a single chloroplast, arguing for the radiative process.

It is plausible that the 730 nm reabsorption-induced emission we observe originates in the peripheral antenna of photosystem I. In our experiment the 730 nm emission is a result of reabsorption of the relatively broad (10–15 nm) 685 nm delayed fluorescence. From prompt fluorescence studies the longer wavelength peaks are generally attributed to photosystem I and the small 710–730 nm emission observed in photosystem II preparations is usually explained as the effect of contamination by photosystem I [16,24]. From a careful study of prompt fluorescence of photosystem I fractions at room temperature and 77 K, Mullet et al. [20] have suggested that the long wavelength emission band of 710–740 nm originates from absorption at 680 nm in the peripheral antenna.

The efficiency of the process seems to be very high since at higher concentrations the 730 nm intensity becomes a significant fraction of the 685 nm intensity. We note here that since in fig.2 the intensities plotted are those measured at the detector and not corrected for absorption, the 730 nm plateau is only apparently greater than that of the 685 nm intensity. High efficiency raises the question of whether this reabsorption induced emission is of functional significance since, presumably, sunlight also induces this 730 nm emission in leaves. Further experiments to determine the role of this process in photosynthesis are warranted.

In summary, our high resolution emission spectra show that long delayed fluorescence in functioning chloroplasts at room temperature is emitted at 685 nm supporting earlier assumptions that delayed fluorescence originates in photosystem II. The 685 nm emission maximum suggests that emission takes place in the photosystem II core complex following reexcitation by charge recombination. Reabsorption of this radiation leads to a second emission feature at 730 nm. That a reab-

sorption-induced process is involved is established by the quadratic concentration dependence of the intensity at low concentrations. We tentatively identify the 730 nm emission as prompt fluorescence associated with the photosystem I peripheral antenna, although the significance of this latter emission is not yet understood.

The sensitivity and resolution of the technique described here allows measurement of delayed fluorescence spectra seconds after excitation. With the added dimension of high resolution spectroscopy, the study of delayed fluorescence, previously confined to kinetics, can provide new information, and be of value especially under physiological conditions of excitation and temperature, and even in *in vivo* studies.

## REFERENCES

- [1] Arnold, W. and Davidson, J.B. (1954) *J. Gen. Physiol.* 37, 677–684.
- [2] Amesz, J. and Van Gorkom, H.J. (1978) *Annu. Rev. Plant Physiol.* 29, 47–66.
- [3] Wermaas, W.F.J., Renger, G. and Dohnt, G. (1984) *Biochim. Biophys. Acta* 764, 194–202.
- [4] Rutherford, W. and Inoue, Y. (1984) *FEBS Lett.* 165, 163–170.
- [5] Hideg, É. and Demeter, S. (1985) *Z. Naturforsch.* 40c, 827–831.
- [6] Sonnefeld, A., Duysens, L.N.M. and Moerdijk, A. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5889–5893.
- [7] Scott, R.Q., Usa, M., Hideg, É. and Inaba, H. XVIth International Conference on Quantum Electronics, 18–21 July 1988, Tokyo, Japan.
- [8] Demeter, S., Vass, I., Hideg, É. and Sallai, A. (1985) *Biochim. Biophys. Acta* 806, 16–24.
- [9] Arnon, D.I. (1949) *Plant Physiol.* 24, 1–15.
- [10] Scott, R.Q., Usa, M. and Inaba, H. (1989) *Appl. Phys. B*, in press.
- [11] Scott, R.Q. and Inaba, H. (1989) *J. Biolum. Chemilum.*, in press.
- [12] Govindjee and Govindjee, R. (1975) in: *Bioenergetics of Photosynthesis* (Govindjee, ed.) pp. 1–50, Academic Press, New York.
- [13] Hipkins, M.F. and Baker, N.R. (1986) in: *Photosynthesis: Energy transduction. A practical approach* (Hipkins, M.F. and Baker, N.R. eds) pp. 51–100, Information Printing Ltd, Oxford.
- [14] Murata, N., Nishimura, M. and Takayama, A. (1966) *Biochim. Biophys. Acta* 126, 234–243.
- [15] Bose, S. (1982) *Photochem. Photobiol.* 36, 725–731.
- [16] Rijgersberg, C.P., Amesz, J., Thielen, A.P.G.M. and Swager, J.A. (1979) *Biochim. Biophys. Acta* 545, 473–482.
- [17] Diner, B.A. and Wollman, F.A. (1979) *Plant Physiol.* 63, 20–25.
- [18] Kramer, H.J.M., Amesz, J. and Rijgersberg, C.P. (1981) *Biochim. Biophys. Acta* 637, 272–277.

- [19] Barret, J. and Thorne, S.W. (1980) *FEBS Lett.* 120, 24-28.
- [20] Mullet, J.E., Burke, J.J. and Arntzen, C.J. (1980) *Plant Physiol.* 65, 814-822.
- [21] Cho, F. and Govindjee (1970) *Biochim. Biophys. Acta* 216, 139-150.
- [22] Harnischfeger, G. (1977) *Adv. Bot. Res.* 5, 1-52.
- [23] Strehler, B.L. and Arnold, W. (1951) *J. Gen. Physiol.* 34, 809-820.
- [24] Franzen, L.-G., Styring, S., Étienne, A.-L., Hansson, Ö. and Vernet, C. (1986) *Photobiochem. Photobiophys.* 13, 15-28.
- [25] Wessels, J.S.C., Van Alphen-van Waveren, O. and Voorn, G. (1973) *Biochim. Biophys. Acta* 292, 741-752.
- [26] Van Dorssen, R.J., Plijter, J.J., Dekker, J.P., Den Ouden, A., Amesz, J. and Van Gorkom, H.J. (1987) *Biochim. Biophys. Acta* 890, 134-143.