In: Garab G. (ed) Photosynthesis: Mechanisms and Effects. Vol. V, pp. 4253-4258. Kluwer Academic Publishers, Dordrecht (1998)

CHLOROPHYLL FLUORESCENCE: NEW INSTRUMENTS FOR SPECIAL APPLICATIONS

Ulrich Schreiber
Julius-von-Sachs Institut für Biowissenschaften, Universität Würzburg,
Julius-von-Sachs Platz 2, D-97082 Würzburg, Germany

Key words: guard-cell chloroplast, microalgae, microscopy, microfibre probe, phytoplankton

1. Introduction

During the past decade there has been remarkable progress in the understanding and practical use of chlorophyll fluorescence in plant science (1,2,3). This progress has resulted from fruitful interactions between three different research disciplines: basic research (dealing with the dynamics of excitation transfer, photochemical charge separation and electron transport), applied research (making use of fluorescence as an noninvasive tool) and the development of new instruments and methodology, to measure fluorescence and to extract the essential information from it. In particular, with the introduction of pulse-amplitude-modulated (PAM) fluorometers and the saturation pulse method of quenching analysis (4), chlorophyll fluorescence has gained widespread applications. It provides manifold information at various levels of the complex process of photosynthesis, starting from light absorption, energy transfer and primary energy conversion, and ending with the export of assimilates from the chloroplast. Due to its large signal amplitude, chlorophyll fluorescence traditionally has been a pioneering tool in photoynthesis research. Making use of recent progress in optoelectronics and microprocessor/computer technology, a new generation of chlorophyll fluorometers with extreme sensitivity and selectivity has been developed, the essential features of which will be outlined in the present communication.

2. Description of Instruments and Results

2.1 *Phytoplankton analysis by 4-wavelength excitation technique*

Since the early work of Lorenzen (5), chlorophyll fluorescence has become increasingly important for assessment of phytoplankton mass and primary productivity (6). Very sensitive techniques have been developed to measure chlorophyll content and to analyze basic parameters of photosynthetic activity in natural surface waters down to 0.1 µg Chl/l (7, 8). The so far available instrumentation has been limited by the fact that it cannot distinguish between different types of phytoplankton, like green algae, diatoms and cyanobacteria. In principle, such distinction is possible on the basis of the specific fluorescence excitation properties of differently pigmented phytoplankton groups (8, 9). A practical version of such a device has been developed (Kolbowski and Schreiber, unpublished) and recently has become generally available (PHYTO-PAM Phytoplankton Analyzer, Heinz Walz GMBH, Effeltrich, Germany).

The PHYTO-PAM employs light-emitting-diodes (LED) to excite chlorophyll fluorescence alternatingly by 10 µs light pulses at four different wavelengths (470, 535,

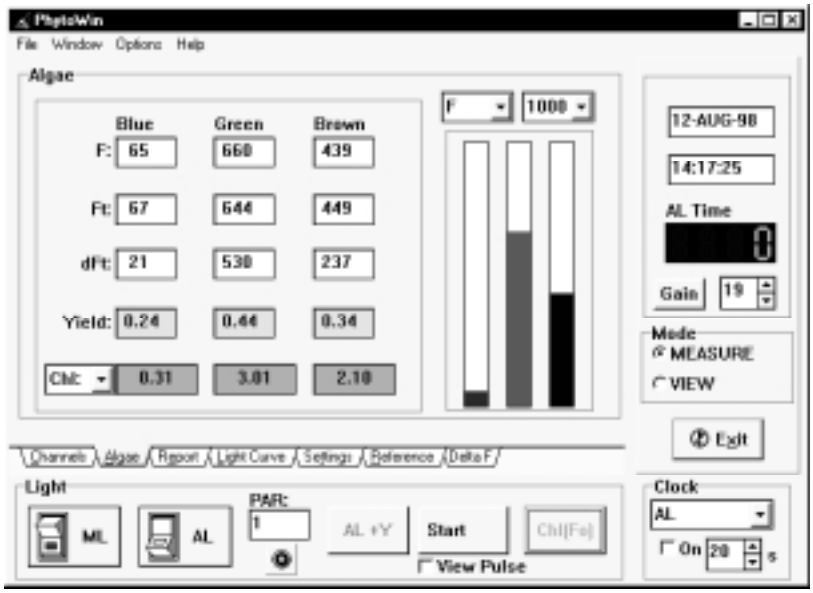

Figure 1
User surface of the PHYTO-PAM with display of „Algae"-window showing deconvoluted values of fluorescence, saturation pulse induced dF, Yield, and chlorophyll concentrations for cyanobacteria, green algae and diatoms in a river-water sample.

620 and 650 nm). The fluorescence pulses are detected by a photomultiplier and amplified under microprocessor-control, resulting in 4 separate continuous signals (4 channels). The fluorometer is operated in conjunction with a Pentium-PC and special Windows software for data deconvolution and analysis. The fluorescence information is displayed in seven different "Windows" on the PC-monitor screen. Fig. 1 shows the user surface and screen-layout with the "Algae"-window being active. Data from a typical measurement, which characterizes the phytoplankton in a river-water sample (Main river) are displayed. The measurement involved a chlorophyll determination (Chl, in µg/l) on the basis of chlorophyll fluorescence yield in the quasi-dark state (Fo) and determination of the quantum yield of photosystem II with the help of a brief pulse of saturating light. When a saturation pulse is triggered, the momentary fluorescence yield (Ft) is sampled and the increase of fluorescence (dF=Fm-Ft) is determined. The quantum yield (Yield) corresponds to dF/Fm. The displayed data for cyanobacteria (Blue), green algae (Green) and diatoms (Brown) were calculated from the original 4-channel fluorescence data by an on-line deconvolution routine, based on previously stored "reference excitation spectra". Such "spectra", which consist of only four points at 470, 535, 620 and 650 nm, can be readily measured under "Reference" for any pure algae culture. The reliability of deconvolution depends on proper choice of reference spectra. However, in any case, the differences between cyanobacteria, green algae and diatoms are sufficiently large to allow at least a coarse differentiation, even if the particular species contained in a sample were not identified.

Fluorescence not only provides information on the content of phytoplankton, but also on its photosynthetic activity, making use of the saturation pulse method. In particular, the effective photosystem II quantum yield observed during continuous illumination is closely correlated with relative electron transport rate (10). Under "Light Curve", the PHYTO-PAM provides a routine for measuring light response curves, which give insight into the light saturation properties and photosynthetic capacity of the differently pigmented algal groups. In Fig.2 the Light Curves corresponding to the sample of Fig.1 are shown. Only the responses of green algae (3.0 µg Chl/l) and of diatoms (2.1 µg Chl/l) are displayed, as the content of cyanobacteria (0.3 µg Chl/l) was too low to give a satisfactory response with a single recording. In the given example, the light responses of green algae and diatoms were very similar, with I_k(green) = 222 and

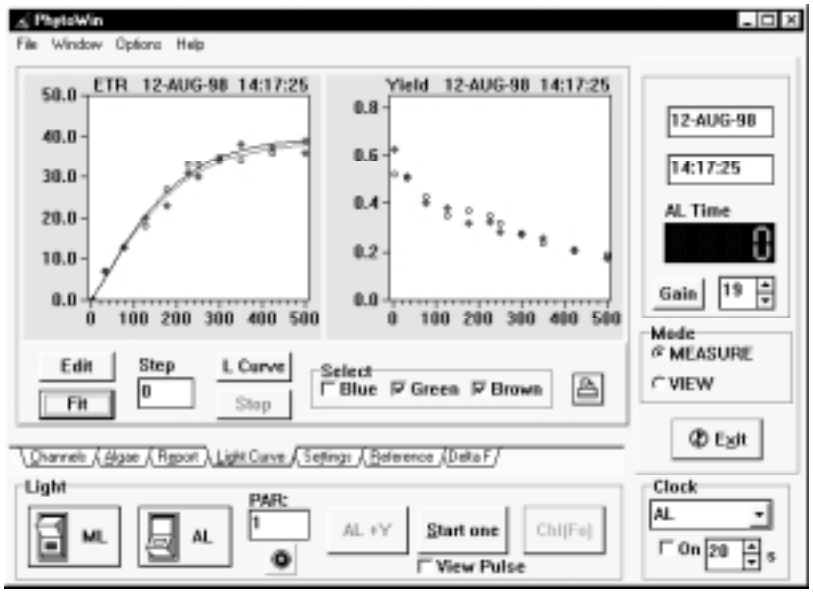

Figure 2
User surface of the PHYTO-PAM in the „Light Curve" mode of operation. Display of light response curves of relative electron transport rate (ETR) and of effective quantum yield, deconvoluted for green algae and diatoms in a river-water sample (see also Figure 1).

I_k(brown) = 212 µmol quanta/m^2s PAR. For comparison, it may be mentioned that a pure culture of *Ankistrodesmus* grown in artificial light was characterized by a much lower value of I_k = 38 µmol quanta/m^2s PAR (original data not shown).

While it is not surprising that, as in the example of Fig.2, green algae and diatoms, having experienced the same light conditions, display very similar light response curves, in other cases one may also encounter samples with different behaviour. An example is given in Fig. 3, which shows light response curves of green algae and diatoms in a water sample from Sydney Harbour. In contrast to the green algae, in this case the diatoms show an unusually low quantum yield in the dark, which rises in low light, before it declines again in parallel to the quantum yield of the green algae. This unusual behaviour of diatoms appears likely to reflect partial reduction of the photosystem II acceptor pool in the dark by electrons from reduced stroma components, possibly via NADPH-dehydrogenase, as previously observed in cyanobacteria (11). As a consequence, in the dark the diatoms may be in state 2 (12), which by illumination would be reversed into state 1.

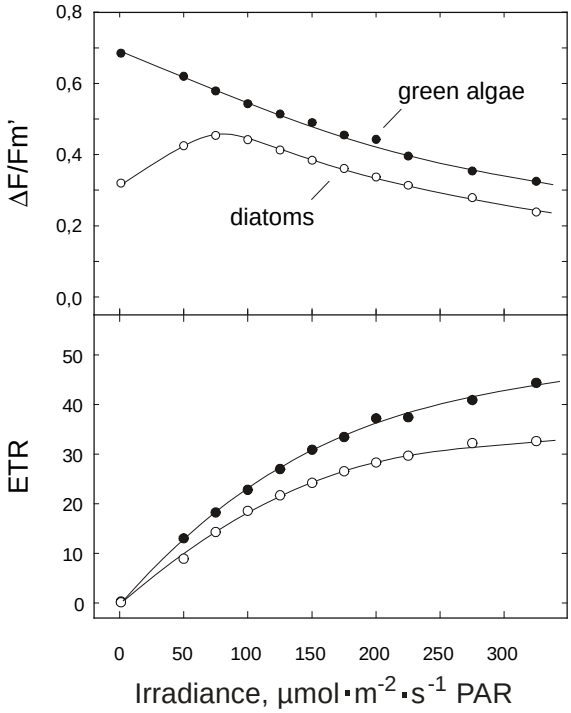

Figure 3 Light response curves of green algae and diatoms in a Sydney Harbour water sample.

With the present optical geometry of the PHYTO-PAM, which essentially has been previously described (9), in a single recording the detection limit is at 0.1 µg Chl/l. The limiting factor is a background signal corresponding to ca. 5 µg Chl/l. Although this background signal can be

readily determined (from measurement with filtrate) and automatically subtracted from the total signal, it determines the overall noise level. The signal/noise and, hence, the detection limit can be improved by signal averaging. A special "Delta F"-mode of operation is provided, in which the ΔF-values induced by repetitive saturation pulses are averaged, with deconvolution into the contributions of green algae, diatoms and cyanobacteria. In this way, independent of any background signal, only the photosynthetically active chlorophyll (characterized by variable fluorescence, ΔF) can be assessed.

2.2 Ultrasensitive measurements at the level of single cells and chloroplasts

Standard PAM fluorometers are equipped with PIN-photodiodes as fluorescence detectors, which display a very large dynamic range, thus tolerating large background signals without any significant increase in noise. While this is of particular advantage in field applications, when fluorescence is measured e.g. in full sun light, it is also essential for use of the saturation pulse method, which employs actinic light pulses ca. 10^4 times more intense than the modulated measuring light. Photomultipliers, on the other hand, while being far more efficient in the detection of low light signals, are strongly disturbed by background light. Very recently, a new generation of PAM fluorometers has been developed (Schreiber and Gademann, unpublished) and now has become generally available, which makes use of the exceptional sensitivity of photomultipliers and still allows quenching analysis by the saturation pulse method (PAM-CONTROL, Universal Control Unit for Ultrasensitive Chlorophyll Fluorescence Measurements, in conjunction with various emitter-detector units, Heinz Walz GmbH). Here a short description of the so-called MICROSCOPY-PAM fluorometer and some examples of the performance of this new device will be presented.

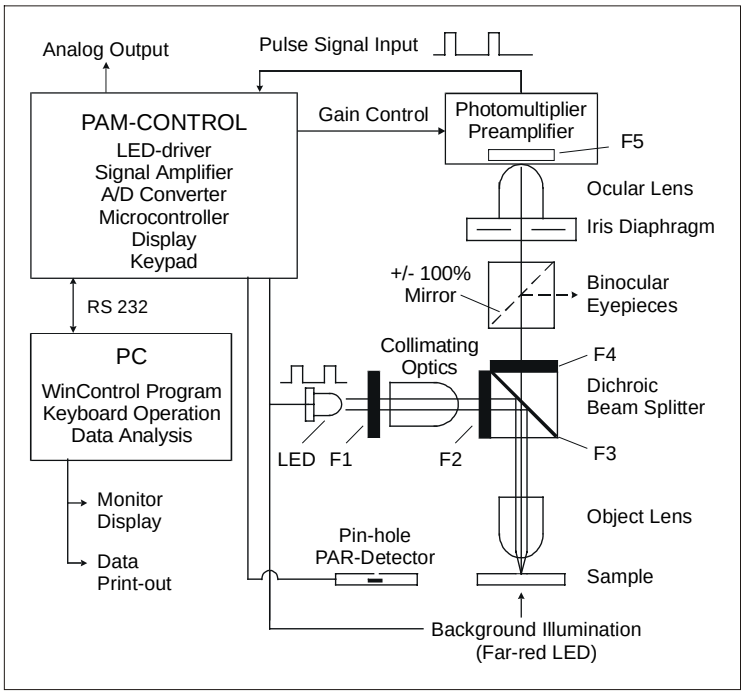

Figure 4 Block diagram of MICROSCOPY-PAM chlorophyll fluorometer. See text for further details.

The MICROSCOPY-PAM consists of a modified epifluorescence microscope, the PAM-CONTROL unit and a Pentium-PC with dedicated Windows-software (WinControl) for system operation and fluorescence analysis. Fig.4 shows a block-diagram of the new device. A blue LED peaking at 470 nm, which is installed in place of the usual Xe-arc lamp in the excitation pathway of the microscope, does not only provide pulse-modulated measuring light, but also serves for actinic and saturation pulse illumination. A miniature photomultiplier is mounted on the phototube adaptor of the microscope. By visual inspection, with the help of an iris diaphragm the field of view can be narrowed

down, such that the fluorescence characteristics of a particular, microscopically small object (as e.g. a single cell or chloroplast) can be selectively assessed.

In Fig.5 typical recordings of dark-light induction curves are displayed, which were measured with the MICROSCOPY-PAM. It is apparent that with a single cell of

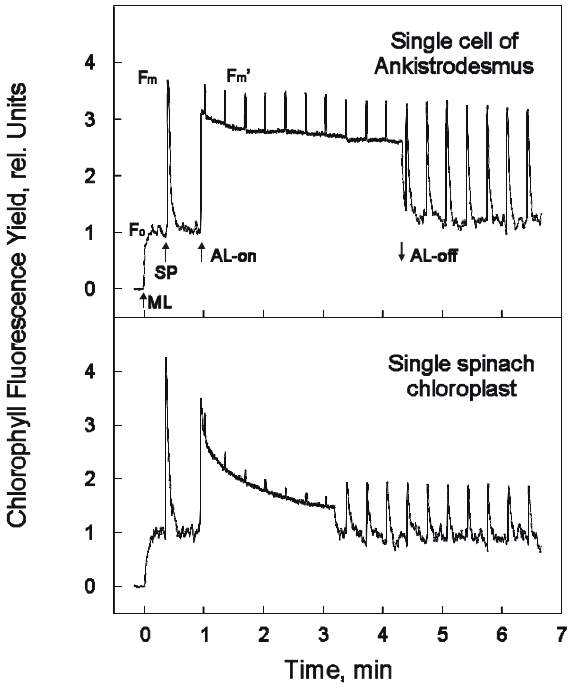

Figure 5 Typical recordings of dark-light induction curves with repetitive application of saturation pulses as measured with the MICROSCOPY-PAM .

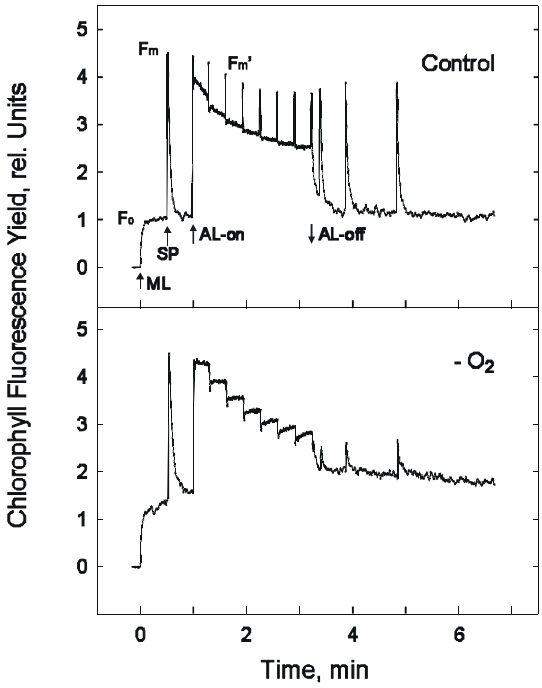

Figure 6 Dark-light induction curves with repetitive saturation pulses for quenching analysis measured on a single guard cell pair of Vicia faba in presence and absence of O_2.

Ankistrodesmus (3x12 µm) and a single spinach chloroplast (ϕ 5 µm) essentially the same fluorescence information can be gained as usually obtained with algae suspensions or whole leaves containing millions of cells. When only the measuring light (ML) is applied, minimal fluorescence yield, Fo, is monitored. Maximal fluorescence, Fm, is induced by a saturation pulse, SP. Following onset of actinic illumination (AL-on), repetitive saturation pulses are applied to assess maximal fluorescence yield, Fm', which is lowered with respect to Fm by nonphotochemical quenching. The WinControl software provides on-line calculation of effective quantum yield and quenching coefficients, which are displayed on the PC monitor screen (not shown). As with the PHYTO-PAM described above (see Fig.2), also a window for recording of light response curves is available (not shown).

An important practical application of the MICROSCOPY-PAM relates to the study of guard-cell photosynthesis. As shown in Fig.6, the fluorescence responses of *Vicia faba* guard cells display all the well-known features of a fully functional photosystem II and also of apparently normal electron transport activity. However, this electron transport does not necessarily reflect Calvin cycle activity and CO_2-fixation. This may be concluded from the fact that it is severely suppressed when molecular oxygen is removed. While further discussion of this interesting aspect would be out of scope of the

present contribution, it may be assumed that O_2-dependent electron flow involving the Mehler-Ascorbate-Peroxidase cycle (13) plays a particular role in guard cell chloroplasts as an effective means of providing ATP for ion-pumping which is essential for stomata functioning.

In conjunction with a fiber-optic microprobe the PAM-CONTROL unit can also be used for ultrasensitive fluorescence measurements in different layers of photosynthetically active material (Schreiber and Gademann, unpublished). A similar, although somewhat less sensitive measuring system based on a standard PAM fluorometer has been previously described for assessment of photosynthesis within leaves (14). The new MICROFIBER-PAM is equipped with four different types of measuring light LEDs (emission peaks at 470, 530, 590 and 650 nm), which can be used alternatively to gain information on differently pigmented organisms, as e.g. green algae, diatoms and cyanobacteria in microbial mats (15).

Acknowledgements

Christof Klughammer, Heinz Reising and Rolf Gademann are thanked for help in the preparation of the manuscript. Thanks are also due to the Heinz Walz GmbH for providing a PHYTO-PAM fluorometer, an epifluorescence microscope and a PAM-Control unit.

References

1 van Kooten, O. and Snel, J. (1990) Photosynthesis Res. 25, 147-150
2 Schreiber, U., Bilger, W. and Neubauer, C. (1994) in Ecophysiology of Photosynthesis (Schulze, E.D. and Caldwell, M.M, eds.) pp. 49-70, Springer, Berlin
3 Dau, H. (1994) Photochem. Photobiol. 60, 1-23
4 Schreiber, U., Schliwa, U. and Bilger, W. (1986) Photosynth. Res. 10, 51-62
5 Lorenzen, C.J. (1966) Deep-Sea Research 13, 223-227
6 Falkowski, P.G. and Kolber, Z. (1995) Aust. J. Plant Physiol. 22, 341-355
7 Kolber, Z. and Falkowski, P.G. (1993) Limnology and Oceanography 38, 1646-1665
8 Schreiber, U., Schliwa, U. and Neubauer, C. (1993) Photosynth. Res. 36, 65-72
9 Kolbowski, J. and Schreiber, U. (1995) in Photosynthesis: from Light to Biosphere (Mathis, P. ed.) pp. 825-828, Kluwer Academic Publishers, Dordrecht, The Netherlands
10 Genty, B., Briantais, J.-M. and Baker, N.R. (1989) Biochim. Biophys. Acta 990, 87-92
11 Mi, H., Endo, T., Schreiber, U. Ogawa, T. and Asada, K. (1992) Plant Cell Physiol. 33, 1233-1237
12 Allen, J.F. (1991) Biochim. Biophys. Acta 1098, 275-335
13 Schreiber, U., Hormann, H., Asada, K. and Neubauer, C. (1995) in Photosynthesis: from Light to Biosphere (Mathis, P. ed.) pp.813-818, Kluwer Academic Publishers, Dordrecht, The Netherlands
14 Schreiber, U., Kühl, M., Klimant, I. and Reising, H. (1996) Photosynth. Res. 47, 103-109
15 Kühl, M. and Jorgensen, BB. (1992) Limnol. Oceanogr. 37, 1813-1823