Measurement of algae PSII photosynthetic parameters using high-frequency excitation flashes

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We establish a system to measure the functional absorption cross section of photosystem II (PSII) (σ_{PSII}) and maximum quantum yield of photochemistry in PSII ($F_{\mathrm{v}}/F_{\mathrm{m}}$). The system utilizes a sequence of high-frequency excitation flashes at microsecond intervals to induce a microsecond-level fluorescence yield curve. Parameters σ_{PSII} and $F_{\mathrm{v}}/F_{\mathrm{m}}$ are calculated by fitting the curve using nonlinear regression. Experimental results show that the relative standard deviation (RSD) of the system is less than 3%, and the correlation coefficient of $F_{\mathrm{v}}/F_{\mathrm{m}}$ values measured by this system and those measured by pulse amplitude modulation method is 0.950.

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Measurement of algae photosynthetic parameters provides theoretical basis for environmental pollution monitoring and algal bloom prediction, and helps to understand marine carbon cycle and marine primary productivity[1−5].

As a non-invasive, convenient, and reliable method, chlorophyll fluorescence method requires no extensive sample manipulation when measuring algae photosynthesis, thus it shows great potential for *in situ* measurement. This method has been well developed and widely used[6−9]. Mauzerall[10] first proposed the pump and the probe (P&P) fluorescence method for photosynthesis measurement. An intense pump flash is used to reduce all the primary electron acceptors Q_{A}, and the change of fluorescence yield is measured by a relatively low intensity probe flash. The probe flash intensity must low enough to avoid actinic effect, so the signal-to-noise ratio (SNR) is low in this method. Based on P&P method, Schreiber[11] proposed pulse amplitude modulation (PAM) method, which permited a high SNR by modulating the measuring light. But still, the modulation frequency must low enough (8−688 Hz) to avoid actinic effect, leading to a low time resolution. Consequently, this method cannot obtain a microsecond-level fluorescence yield curve, which allows the calculation of functional absorption cross section of photosystem II (PSII) (σ_{PSII}). The fast repetition rate (FRR) method put forward by Kolber and Falkowski provides a solution for the above problems[12]. This method applies a sequence of excitation flashes at microsecond intervals to induce a microsecond-level fluorescence yield curve by reducing the primary electron acceptor Q_{A}, and parameters σ_{PSII} and $F_{\mathrm{v}}/F_{\mathrm{m}}$ can be retrieved by fitting the induced curve. Based on the theory of FRR method, we established a PSII photosynthetic parameter measurement system using high-frequency excitation flashes at microsecond intervals, which permits the measurement of microsecond-level fluorescence yield curve and the calculation of σ_{PSII} and $F_{\mathrm{v}}/F_{\mathrm{m}}$.

In PSII, the quanta of excitation light are absorbed and transferred to reaction centers, and the overall efficiency of light trapping and energy transfer is described by the functional absorption cross section σ_{PSII}. The arrived excitation energy oxidizes reaction centre pigment P680 and reduces primary electron acceptor Q_{A} to Q_{A}^-, leading to the closure of PSII reaction center and then an increase of the fluorescence yield. Only after the reoxidation of Q_{A}^- (i.e., electron transfer from Q_{A}^- to plastoquinone (PQ) pool), can the reaction center reopen, and the fluorescence yield decline.

The fluorescence yield is determined by excitation energy, σ_{PSII}, and the redox state of Q_{A}. When excited by high-frequency excitation flashes at short enough intervals, all the Q_{A} can be reduced without any reoxidation. In such a situation, the fluorescence yield can be described as[13,14]

$$F_i = F_{\mathrm{o}} + (F_{\mathrm{m}} - F_{\mathrm{o}})A_i = F_{\mathrm{o}} + F_{\mathrm{v}}\left[1 - \exp\left(-\sigma_{\mathrm{PSII}} \sum_{j=1}^{j=i} l_j\right)\right], \tag{1}$$

where F_{o} is the minimal fluorescence yield measured when all PSII reaction centers are open, F_{m} is the maximum fluorescence yield measured when all PSII reaction centers are closed, F_{v} is variable fluorescence (i.e., the difference between the maximum and minimal fluorescence yield), $A_i (0 \leqslant A_i \leqslant 1)$ is the fraction of PSII reaction centers closed at a given state of the excitation, and l_j is the energy of the jth flash.

To determine the photosynthetic parameters, we need to record the excitation flashes and the corresponding fluorescence signals, which are used to calculate the fluorescence yield curve. The fluorescence yield curve is then fitted into Eq. (1) using nonlinear regression to calculate σ_{PSII}, F_{o}, F_{v}, and $F_{\mathrm{v}}/F_{\mathrm{m}}$. σ_{PSII} is related to the type of

algae and the wavelength of the excitation light. In this letter, the discussion was limited to the measurements of σ_{PSII} for green algae (*chlorella pyrenoidosa*) using 468-nm excitation LED.

The schematic of the measurement system is shown in Fig. 1. The system is composed of 468-nm LED array, LED driver unit, sample cell, detection unit for fluorescence signal and LED excitation light (using as reference signal), digital oscilloscope, and computer. To uniformly illuminate the sample cell, the 10 LEDs are ranged as a cycle around the sample cell, and low-pass glass filters are used to eliminate the long-wavelength radiation from the LEDs[15]. A microcontrol unit (MCU) is utilized to generate modulated signal with 2-μs duration at 1-μs intervals, which is then used to modulate the LEDs through a LED driver unit consist of TC4422A and MOSFET IRF640. The LEDs illuminate the sample cell with an average flux of 30000 μmol quanta/m^2/s, and the excitation energy is provided by 7 discharge capacitors.

The induced 685-nm fluorescence is collected from the bottom of the sample cell by a photomultiplier (PMT) with a 685-nm band-pass interference filter and a 600-nm long-pass glass filter[16]. The excitation light is detected by a PIN photodiode. The two signals are sampled by a digital oscilloscope with a sample rate of 20 MS/s and transferred to a computer. The fluorescence curve is calculated, and consequently the PSII parameters are retrieved on the computer.

The excitation light and stimulated fluorescence of healthy *chlorella pyrenoidosa* sampled by the digital oscilloscope are shown in Figs. 2 and 3, respectively. The fluorescence yield is the ratio of the emitted fluorescence energy to the excitation energy absorbed by chlorophyll, but the measurement of the accurate absorbed excitation energy will greatly increase the complexity of the system and introduce more uncertain factors, hence the excitation energy is used instead. The integrations LED_i and Fluo_i are used as the energy of excitation flash and the energy of the corresponding fluorescence calculated from

$$\mathrm{LED}_i = \int_{t_i}^{t_i+\Delta t} l(t)\mathrm{d}t, \tag{2}$$

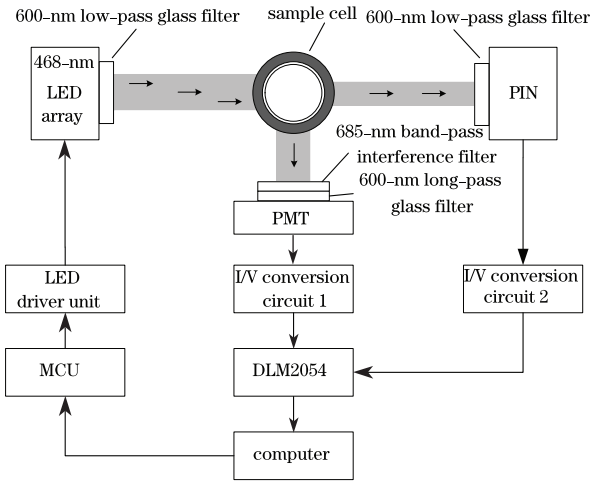

Fig. 1. Schematic of the photosynthetic parameter measurement system using high-frequency excitation flashes.

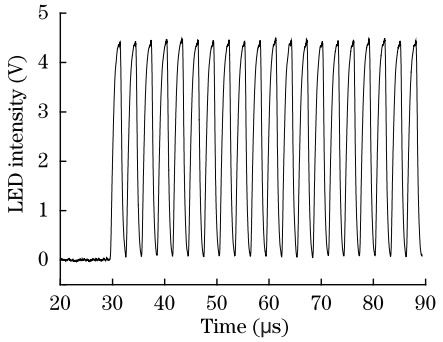

Fig. 2. Excitation LED flash signal.

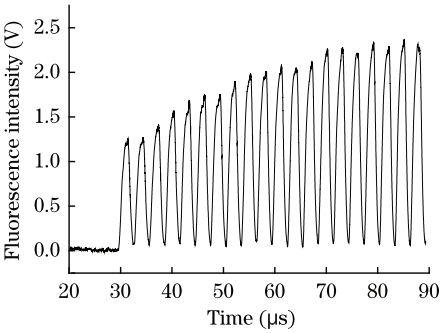

Fig. 3. Fluorescence signal corresponding to the excitation LED flashes.

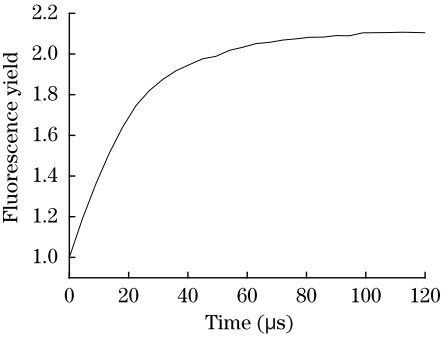

Fig. 4. Fluorescence yield of healthy *chlorella pyrenoidosa.*

$$\mathrm{Fluo}_i = \int_{t_i}^{t_i+\Delta t} f(t)\mathrm{d}t, \tag{3}$$

where Δt is the duration of the excitation flash. Consequently, the fluorescence yield is obtained from

$$F_i = \frac{\mathrm{Fluo}_i}{\mathrm{LED}_i}. \tag{4}$$

Obviously, the fluorescence yield F_i (Fig. 4) calculated from Eqs. (2)–(4) is different from the actual value F_i' by a coefficient m. Equation (1) suggests these this coefficient results in a same coefficient on F_{o}, F_{v}, and F_{m}, but not σ_{PSII}. Noticing that the maximum quantum yield of photochemistry in PSII ($F_{\mathrm{v}}/F_{\mathrm{m}}$) is a ratio, which eliminates the influence of coefficient m, the parameters we concern (i.e., σ_{PSII} and $F_{\mathrm{v}}/F_{\mathrm{m}}$) are not influenced by this coefficient.

F_i, F_{o}, and F_{v} are all dimensionless, and the units of σ_{PSII} and actual excitation energy l_j are respectively

$Å^2$/quanta and quanta/$Å^2$. To determine the actual excitation energy l_j that is needed in the calculation of the photosynthetic parameters in Eq. (1), we established a relationship between the integration LED_0 and the actual excitation flash energy l_0 (quanta/$Å^2$) when calibrating the system, shown as

$$l_0 = k * \mathrm{LED}_0, \tag{5}$$

where l_0 is measured by a PAR sensor.

During the retrieve, the actual excitation flash energy l_j is obtained from LED_j using Eq. (5). F_o, F_v, and σ_PSII are then calculated by fitting the measured fluorescence yield curve into Eqs. (1)–(4) using nonlinear regression. The values of F_o, F_v, σ_PSII, and $F_\mathrm{v}/F_\mathrm{m}$ calculated from the curve shown in Fig. 4 are respectively 0.756, 1.451, 258.57 $Å^2$/quanta, and 0.657.

The main interference in the fluorescence yield measurement and photosynthetic parameters retrieve is the intensity fluctuation of the excitation flash, which consists of the intensity fluctuation between flashes in one measuring process and the intensity fluctuation between different measuring processes because of the excitation light source aging and etc.

The fluorescence yield F_i is calculated as the ratio of the fluorescence energy Fluo_i and the excitation energy LED_i, and Fluo_i is in proportion to LED_i, thus F_i is not influenced by both of the two intensity fluctuations, and in consequence F_o and F_v are both immune to the intensity fluctuations according to Eq. (1). But it is not the same for σ_PSII because it is in inverse proportion to $\sum_{j=1}^{j=i} l_j$ as shown in Eq. (1). The intensity fluctuation between different measuring processes would do great harm to the accuracy of σ_PSII. The influence of this intensity fluctuation was eliminated by intensity correction. The excitation energy measured in the system calibration was used as a standard to correct this intensity fluctuation during the measurement. 8 parallel measurements of healthy *chlorella pyrenoidosa* were implemented and the results were shown in Table 1. The relative standard deviations (RSDs) of σ_PSII and $F_\mathrm{v}/F_\mathrm{m}$ were respectively 2.96% and 1.62%, indicating a good stability.

Excessive Cu^{2+} can damage the thylakoid membrane of algal cells, and inactivate the PSII reaction centers by inhibiting the electron transfer[17,18], which cause a change of the algae photosynthesis state. Therefore, thevalidity of the measurement system was analyzed by utilizing it to measure the photosynthetic parameters of the *chlorella pyrenoidosa* stressed by Cu^{2+}.

The concentrations of *chlorella pyrenoidosa* and Cu^{2+} were respectively 100 μg/L and 25 μmol/L. The measurement was implemented every 5 min. With the increase of stress time, the rate of fluorescence yield saturation and the maximum fluorescence yield F_m significantly declined (Fig. 5), and the retrieved σ_PSII (Fig. 6) and $F_\mathrm{v}/F_\mathrm{m}$ (Fig. 7) also markedly declined.

The declines of σ_PSII and the rate of fluorescence yield saturation indicated the damage of thylakoid membrane. As the light absorption and energy transfer were implemented on the thylakoid membrane, the damage of the thylakoid membrane caused the decline of σ_PSII, which resulted in a decrease of excitation energy arrived at reaction centers and in consequence a decline of the rate of fluorescence yield saturation.

The declines of F_m and $F_\mathrm{v}/F_\mathrm{m}$ indicated the inactivation of PSII reaction centers. There was no photochemical action in inactivated reaction centers, and the arrived excitation energy dissipated as heat[19]. Thus the fluorescence yield of the inactivated reaction centers remained F_o even under ambient light, causing the decline of F_m and consequently the decline of $F_\mathrm{v}/F_\mathrm{m}$.

As a comparison, the Water PAM instrument based on method was employed to measure $F_\mathrm{v}/F_\mathrm{m}$[20]. The Water PAM can only measure $F_\mathrm{v}/F_\mathrm{m}$, but not σ_PSII. The correlation coefficient of the $F_\mathrm{v}/F_\mathrm{m}$ values measured by the system and those measured by Water PAM was 0.950 (Fig. 8). We also notice that the average value of $F_\mathrm{v}/F_\mathrm{m}$ measured by the system for healthy *chlorella pyrenoidosa* is 0.655, which is almost the same as the normal value (0.65)[21].

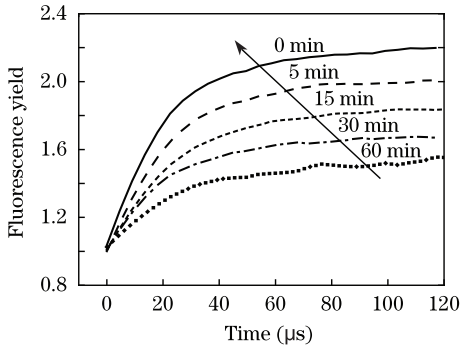

Fig. 5. Fluorescence yield saturation curves with the stress time increasing from 0 to 60 min.

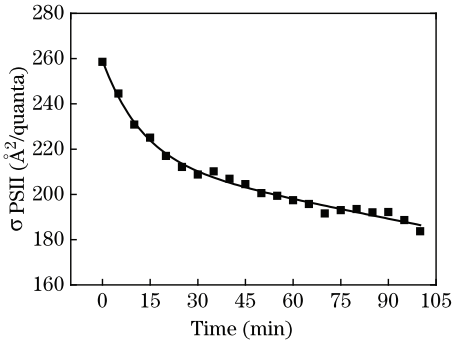

Fig. 6. Measured σ_PSII values of *chlorella pyrenoidosa* versus increasing stress time.

Table 1. Parallel Measurement Results of Healthy Chlorella Pyrenoidosa

No. of Samples	1	2	3	4	5	6	7	8
σ_PSII ($Å^2$/quanta)	258.57	269.12	250.36	248.77	260.29	259.87	250.26	266.46
$F_\mathrm{v}/F_\mathrm{m}$	0.657	0.645	0.639	0.670	0.651	0.662	0.649	0.665

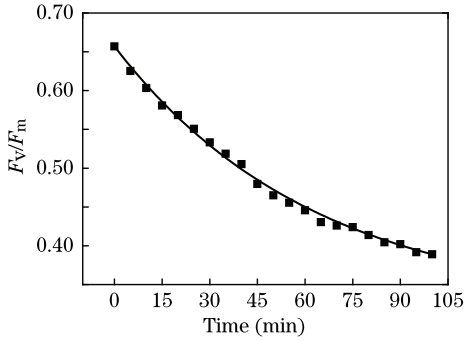

Fig. 7. Measured F_v/F_m values of *chlorella pyrenoidosa* versus increasing stress time.

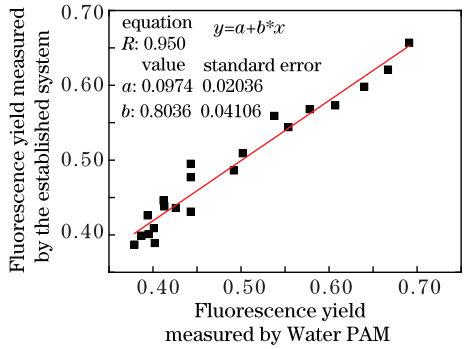

Fig. 8. (Color online) Correlation analysis of F_v/F_m values measured by the established system and Water PAM in the Cu^{2+} stress experiment.

In conclusion, we establish a PSII photosynthetic parameter measurement system using high-frequency excitation flashes. The system permits the induction of microsecond-level fluorescence yield curve, and calculates σ_{PSII} and F_v/F_m by fitting the fluorescence yield curve using nonlinear regression. The correlation coefficient of the F_v/F_m values measured by this system and those measured by Water PAM method is 0.950. Furthermore, this system is extensible to induce fluorescence curves related to the electron transfer states of Q_A to PQ pool and PQ pool to PSI, revealing more photosynthesis details.

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