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快速光脉冲藻类光合作用测量方法的激发条件研究

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摘 要：激发光强和激发持续时间是快速光脉冲藻类光合作用测量方法的关键实验条件.通过光脉冲激发实验，定量分析了不同平均激发光强下还原态初级电子受体的比例和发生再氧化的初级电子受体的比例.结果表明：快速光脉冲激发的最佳平均光强为 30 000 μmol quanta·m^{-2}·s^{-1}，最佳激发持续时间为 70 μs；30 000 μmol quanta·m^{-2}·s^{-1}平均激发光强能够在 70 μs 内还原 96.08%的初级电子受体，且仅9.81%的初级电子受体发生了再氧化.

关键词：藻类；光合作用；荧光；电子传递；光学测量
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Determining the Optimal Excitation Condition of High-frequency Flash Method for Algae Photosynthetic Parameters Measurement

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Abstract: The excitation intensity and excitation duration are the essential experimental conditions of the high-frequency flash method for the measurement of algae photosynthetic parameters. The fraction of reduced primary electron acceptors and the reoxidation extent of these reduced primary electron acceptors were analyzed under different excitation intensities and excitation durations. The analysis results indicate that 30 000 μmol quanta·m^{-2}·s^{-1} is the optimal excitation intensity and 70 μs is the optimal excitation duration. Under this optimal excitation condition, 96.08% of the primary electron acceptors are reduced and only 9.81% primary electron acceptors reoxidize.

Key words: Algae; Photosynthesis; Fluorescence; Electron transfer; Optical measurement
OCIS Codes: 170.1420; 280.4788; 120.0120; 300.6280

0 Introduction

The chlorophyll *a* fluorescence yield can be used to access the algae photosynthesis activity. Many methods have been developed based on this principle [1-5], including Pump and Probe (P&P) method[6] and Pulse Amplitude Modulation (PAM) method[7]. While a high-frequency flash method put forward by Kolber in the 1990′s[8-9] is much more promising because it eliminates the influence of the reoxidation of reduced primary electron acceptors, improves the retrieval accuracy and realizes the measurement of σ_{PSII}. This

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method uses a sequence of high-frequency excitation flashes at microsecond intervals to reduce all the primary electron acceptors, inducing a microsecond-level fluorescence yield curve. The curve is then fitted by fluorescence yield model to calculate the PSII functional absorption cross section σ_{PSII} and PSII maximum quantum yield of photochemistry F_v/F_m[10].

The accuracy of the high-frequency flash method deponds on weather the flashes can reduce all the primary electron acceptors with negligible reoxidation of reduced primary electron acceptors. Therefore, it's critical to choose appropriate excitation intensity and duration. This paper analyzes the fraction of reduced primary electron acceptors and the reoxidation extent of these reduced primary electron acceptors under different average excitation intensities, and determines the optimal excitation intensity and duration.

1 Experimental system

As shown in Fig. 1, the experimental system is composed of LED array and the driver unit, sample cell, detection unit for fluorescence signal and LED excitation light (using as reference signal), digital oscilloscope and computer. The excitation light from the LED array is modulated to flashes of 2 μs duration at 1 μs intervals using modulated signal that is generated by MCU ADuC841. The excitation intensity is controlled by changing the LED current. The induced fluorescence is collected by a PMT (photomultiplier) in 90° direction to the excitation light, and the excitation light is detected by a PIN photodiode. The two signals are sampled by a digital oscilloscope DLM2054 with a sample rate of 25 MS/s and transferred to a computer. The fluorescence curve is calculated, and consequently the photosynthetic parameters are retrieved on the computer.

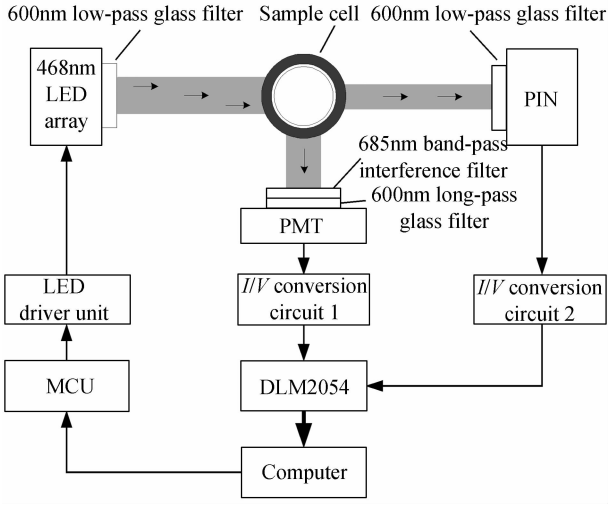

Fig. 1 Experimental system based on high-frequency flash method for photosynthetic parameter measurement

2 Method and analysis

The energy transfer process of algae photosynthesis is shown in Fig. 2. Following the flash excitation, the primary electron acceptor Q_A gets electron and is reduced to Q_A^-, leading to an increase in the fluorescence yield. However, in the flash intervals, Q_A^- transfers electron to Q_B and reoxidizes to

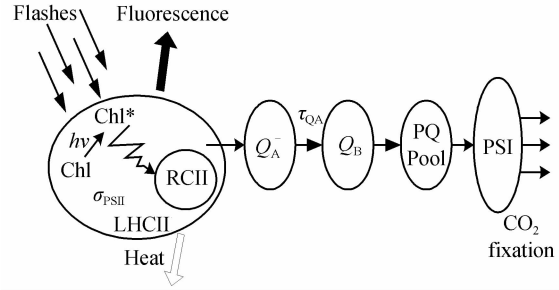

Fig. 2 Energy transfer diagram of algae photosynthesis

Q_A, leading to a decrease of the fluorescence yield. The time constant of Q_A^- reoxidation τ_{QA} is several hundreds of microseconds (100 ~ 300 μs)[11], which is called single turnover period of Q_A. The induced fluorescence yield curve in the high-frequency flash method is described by Eq. 1, from which σ_{PSII} and F_v/F_m can be calculated.

$$F_i = F_o + (F_m - F_o)A_i = F_o + F_v[1 - \exp(-\sigma_{PSII}\sum_{j=1}^{j=i} l_j)] \quad (1)$$

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 reduced primary electron acceptors at a given state of the excitation, and l_j is the energy of the j-th flash.

Eq. 1 is a fluorescence yield model that requires the Q_A^- reoxidation is negligible, and the fluorescence yield approaches F_m[8-9]. The requirements of Eq. 1 can be met by reducing all the primary electron acceptors within the single turnover period, because there's few Q_A^- reoxidation in the single turnover period of Q_A. In this way, the fluorescence yield can approach F_m by reducing all the primary electron acceptors while the Q_A^- reoxidation is negligible.

The high-frequency method with appropriate excitation intensity and duration can reduce all the primary electron acceptors within the single turnover period of Q_A. The determination of excitation intensity and duration can be achieved by analyzing A_i and e_t, which are respectively the fraction of reduced primary electron acceptors and the reoxidation extent of these reduced primary electron acceptors. A_i and e_t can be

calculated from Eq. 2 and Eq. 3[10]. These two equations indicate that the values of l_i, σ_{PSII} and τ_{QA} should be determined before calculating A_i and e_t. l_i can be determined by the reference signal during the measurement, but extra experiments are required to determine σ_{PSII} and τ_{QA}.

$$A_i = A_{i-1}\exp(-\Delta t/\tau_{QA}) + [1-\exp(-\sigma_{PSII} l_i)] \cdot [1-A_{i-1}\exp(-\Delta t/\tau_{QA})] = 1-\exp(-\sigma_{PSII} l_i) \cdot [1-A_{i-1}\exp(-\Delta t/\tau_{QA})] \quad (2)$$

$$e_t = \sum_{i=1}^{i} A_i \exp(-\Delta t/\tau_{QA}) \quad (3)$$

3 Experiments

The single turnover period of Q_A varies between 100 ~ 300 μs according to the algae class and photosynthetic activity. To ensure all the primary electron acceptors are reduced within the single turnover period in any condition, we analyzed the changing process of A_i and e_t in 100 μs. Based on the analysis results, the optimal excitation intensity and duration were determined. According to Eq. 2 and Eq. 3, the values of σ_{PSII} and τ_{QA} are needed for calculations of A_i and e_t. Thus, we first designed experiments to determine σ_{PSII} and τ_{QA}. Then excitation experiments were implemented for the determination of the optimal excitation intensity and duration. The algae sample used in all the experiments in this paper was *chlorella pyrenoidosa*, with a concentration of 80 μg/L. The excitation wavelength was 468nm. The concentration of DCMU solution was 100 μmol/L.

3.1 σ_{PSII} measurement

σ_{PSII} can be calculated from Eq. 1 when the Q_A^- reoxidation is negligible. In the σ_{PSII} measurement experiment, we employed DCMU to inhibit the Q_A^- reoxidation[12-14] for the measurement of σ_{PSII}. 1 mL DCMU solution was added to 100 mL *chlorella pyrenoidosa* solution, and all the primary electron acceptors were reduced by excitation flashes with an average excitation intensity of 10 000 μmol quanta · m^{-2} · s^{-1}. As the Q_A^- reoxidation was inhibited by DCMU, there was no special requirement for the excitation intensity[15]. The induced fluorescence yield curve was shown in Fig. 3, and σ_{PSII} was calculated to be 310Å^2 by fitting the curve to Eq. 1.

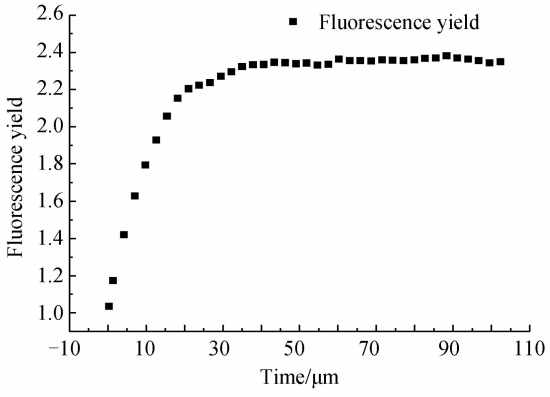

Fig. 3 Fluorescence yield curve measured with the presence of DCMU

3.2 τ_{QA} measurement

A part of primary electron acceptors are reduced when healthy algae are excited by intense flashes, and consequently the fluorescence yield increases to F_t. When the excitation stops, the fluorescence yield begins to decrease because of the reoxidation of the reduced primary electron acceptors. The decay curve is described by Eq. 4 [16-17], and τ_{QA} can be calculated from this curve.

$$F_t = F_o + (F - F_o)\exp(-t/\tau_{QA}) \quad (4)$$

In the τ_{QA} measurement experiment, the algae sample was excited by high-frequency flashes of 2μs duration at 1 μs intervals for 40 μs, and the average intensity was 30 000 μmol quanta · m^{-2} · s^{-1}. Then weak modulated measuring light was employed to record the decay curve of the fluorescence yield, as shown in Fig. 4. The measuring light was modulated pulses of 0.3μs duration at 60 μs intervals. The average intensity of the measuring light was 3 μmol quanta · m^{-2} · s^{-1}, which was so weak that the actinic effect was negligible[18]. Finally, τ_{QA} was calculated to be 223 μs by fitting the recorded decay curve to Eq. 4.

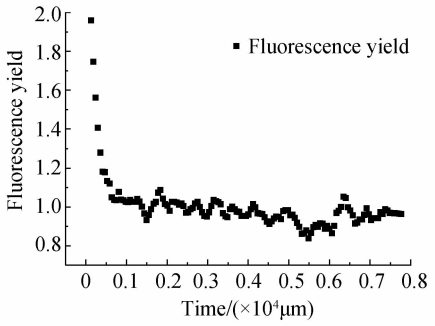

Fig. 4 Decay curve of fluorescence yield following intense excitation flashes

3.3 Excitation experiment

We implemented excitation experiments using a series of excitation intensities to determine the optimal excitation intensity and duration. The average excitation intensities we employed were 5 000, 10 000, 15 000, 20 000 and 30 000 μmol quanta · m^{-2} · s^{-1}. The corresponding fluorescence yield curves are shown in Fig. 5. A_i curves calculated using Eq. 2 are shown in Fig. 6.

As shown in Fig. 5 and Fig. 6, the fluorescence yield curve and A_i curve increased more rapidly with higher excitation intensity. After 100 μs of excitation, the fluorescence yield curves and A_i curves under average excitation intensities of 5 000 ~ 20 000 μmol

quanta • m^{-2} • s^{-1} still had rising trend, and did not achieve the maximum. But when the average excitation intensity was 30 000 μmol quanta • m^{-2} • s^{-1}, both the fluorescence yield curve and A_i curve achieved the maximum at 100 μs, and A_i reached up to 96.46%, which meant that almost all of the primary electron acceptors were reduced.

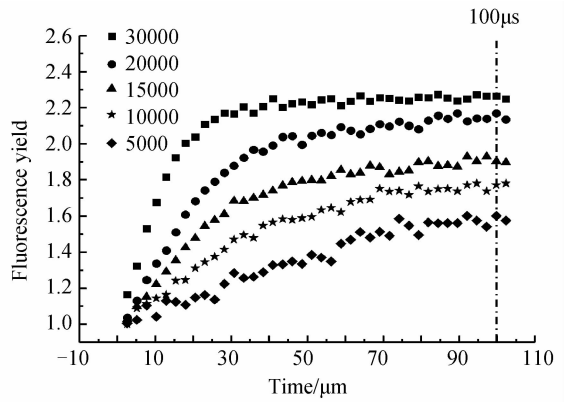

Fig. 5 Fluorescence yield curves under different average excitation intensities

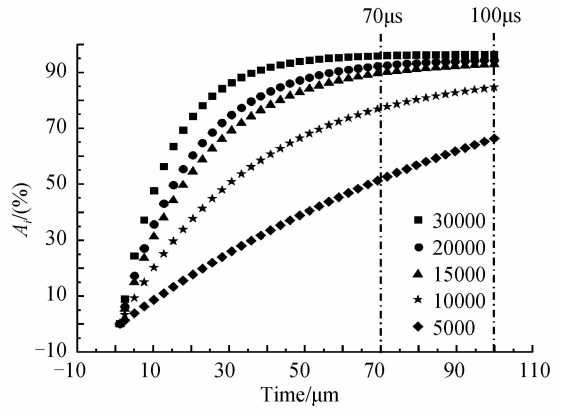

Fig. 6 Fractions of the reduced primary electron acceptors under different average excitation intensities

In order to determine the optimal excitation duration, we further analyzed the change trend of A_i and e_t under 30 000μmol quanta • m^{-2} • s^{-1} average excitation intensity excitation, as shown in Fig. 7. A_i reached up to 96.08% at 70 μs and increased to

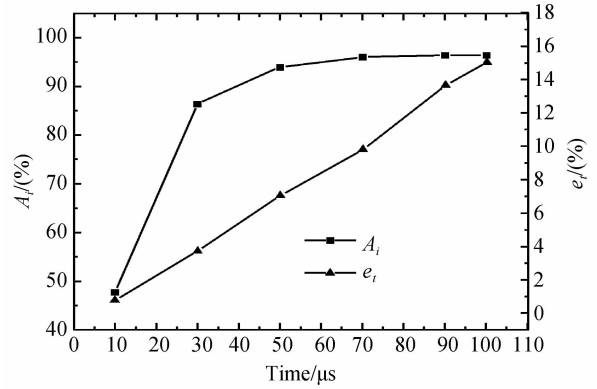

Fig. 7 A_i and e_t versus time under an average excitation intensity of 30 000 μmol quanta • m^{-2} • s^{-1}

96.46% at 100 μs, and the increment per microsecond was only 0.013%. e_t were 9.67% and 15.04% respectively at 70 μs and 100 μs, and the increment per microsecond was 0.17%, which was 13 times as high as that of A_i. The analysis showed that A_i nearly reached the maximum at 70 μs and kept stable, but e_t increased rapidly from 70 μs to 100 μs. Thus the excitation should be stopped as soon as A_i nearly reached to the maximum at 70 μs to avoid more Q_A^- reoxidation and reduction.

The above analysis of A_i and e_t indicated that when the average excitation intensity was 30 000 μmol quanta • m^{-2} • s^{-1}, almost all of the primary electron acceptors were reduced (A_i = 96.08%) within 70 μs, while e_t was just 9.67%, which was negligible[10]. Therefore, we chose 30 000 μmol quanta • m^{-2} • s^{-1} as the optimal average excitation intensity, and 70 μs as the optimal excitation duration. It is necessary to point out that all the primary electron acceptors can be reduced within a shorter time with higher excitation intensity, but higher excitation intensity would lead to non-photochemical fluorescence quenching caused by either carotenoid triplets formation[19] or $P680^+$ accumulation[20], which would influence the retrieval accuracy.

4 Conclusion

In conclusion, we present a method to determine the optimal excitation intensity and duration of the high-frequency flash method for the measurement of algae photosynthetic parameters. The requirements of excitation intensity and duration are specified by analyzing the fluorescence yield model used in the high-frequency flash method. Then the optimal average excitation intensity and excitation duration are respectively determined to be 30 000 μmol quanta • m^{-2} • s^{-1} and 70 μs based on the analysis results of the fraction of reduced primary electron acceptors and the reoxidation extent of these reduced primary electron acceptors. Under this optimal excitation condition, 96.08% of the primary electron acceptors are reduced and only 9.81% primary electron acceptors reoxidize. For different optical structures and excitation protocols, the requirements of excitation intensity and duration may be different, but the analysis method in this paper is universal and can still be employed for the determination of excitation intensity and duration.

References

[1] HAN Bo-ping, HAN Zhi-guo, FU Xiang. Algae photosynthesis: mechanism and model[M]. Beijing: Science Press, 2003.

[2] SUGGETT D J, PRÁŠIL O, BOROWITZKA M A, BOROWITZKA, *et al*. Chlorophyll a fluorescence in aquatic sciences: methods and applications[M]. Springer, 2011.

[3] PAPAGEORGIOU G C, TSIMILLI-MICHAEL M, STAMATAKIS K. The fast and slow kinetics of chlorophyll a fluorescence induction in plants, algae and cyanobacteria: a viewpoint[J]. *Photosynthesis Research*, 2007, **94**(2-3): 275-290.

[4] SERÔDIO J. A chlorophyll fluorescence index to estimate short-term rates of photosynthesis by intertidal microphytobenthos[J]. *Journal of Phycology*, 2003, **39**(1): 33-46.

[5] BAKER N R. Chlorophyll fluorescence: A probe of photosynthesis in vivo[J]. *Annual Review of Plant Biology*, 2008, **59**: 89-113.

[6] MAUZERALL D. Light-induced fluorescence changes in Chlorella, and the primary photoreactions for the production of oxygen [J]. *Proceedings of the National Academy of Sciences*, 1972, **69**(6): 1358-1362.

[7] SCHREIBER U. Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer [M]. Current topics in photosynthesis. Springer Netherlands, 1986: 259-270.

[8] KOLBER Z S, PRÁŠIL O, FALKOWSKI P G. Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols [J]. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1998, **1367**(1): 88-106.

[9] FALKOWSKI P G, KOLBER Z S. Fast repetition rate (FRR) fluorometer and method for measuring fluorescence and photosynthetic parameters: U S 5426306[P]. 1995-06-20.

[10] SHI Chao-yi, ZHANG Yu-jun, YIN Gao-fang, *et al*. Measurement of algae PSII photosynthetic parameters using high-frequency excitation flashes[J]. *Chinese Optics Letters*, 2014, **12**(8): 080101.

[11] KOLBER Z S, FALKOWSKI P G. Multiple protocol fluorometer and method: US,6121053[P]. 2000-9-19.

[12] FALKOWSKI P, KIEFER D A. Chlorophyll a fluorescence in phytoplankton: relationship to photosynthesis and biomass [J]. *Journal of Plankton Research*, 1985, **7**(5): 715-731.

[13] BISWAL A K, DILNAWAZ F, DAVID K A V, *et al*. Increase in the intensity of thermoluminescence Q-band during leaf ageing is due to a block in the electron transfer from QA to QB[J]. *Luminescence*, 2001, **16**(5): 309-313.

[14] VREDENBERG W, PRÁŠIL O. On the polyphasic quenching kinetics of chlorophyll a fluorescence in algae after light pulses of variable length[J]. *Photosynthesis Research*, 2013, **117**(1-3): 321-337.

[15] FALKOWSKI P, KIEFER D A. Chlorophyll a fluorescence in phytoplankton: relationship to photosynthesis and biomass [J]. *Journal of Plankton Research*, 1985, **7**(5): 715-731.

[16] BELYAEVA N E, SCHMITT F J, STEFFEN R, *et al*. PS II model-based simulations of single turnover flash-induced transients of fluorescence yield monitored within the time domain of 100 ns-10 s on dark-adapted Chlorella pyrenoidosa cells[J]. *Photosynthesis Research*, 2008, **98**(1-3): 105-119.

[17] WAN Wen-bo, HUA Deng-xin. Laser-induced chlorophyll fluorescANce lifetime measurement and characteristic analysis [J]. *Acta Physica Sinica*, 2013, **62**(19).

[18] LIU Jing, LIU Wen-qing, ZHAO Nan-jing, *et al*. Phytoplankton chlorophyll fluorescence characteristics excited by various light qualities and intensities [J]. *Acta Optica Sinica*, 2013, **09**: 290-297.

[19] SOMMEVELD A, RADEMAKER H, DUYSENS L N M. Transfer and trapping of excitation energy in Photosystem II as studied by chlorophyll a fluorescence quenching by dinitrobenzene and carotenoid triplet. The matrix model[J]. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1980, **593**(2): 272-289.

[20] DEPREZ J, DOBEK A, GEACINTOV N E, *et al*. Probing fluorescence induction in chloroplasts on a nanosecond time scale utilizing picosecond laser pulse pairs[J]. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1983, **725**(3): 444-454.