Photosynth Res (2017) 133:63–73
DOI 10.1007/s11120-017-0367-x

REVIEW

Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions

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Received: 25 October 2016 / Accepted: 2 March 2017 / Published online: 10 March 2017

Abstract Cyanobacteria are photosynthetic prokaryotes and widely used for photosynthetic research as model organisms. Partly due to their prokaryotic nature, however, estimation of photosynthesis by chlorophyll fluorescence measurements is sometimes problematic in cyanobacteria. For example, plastoquinone pool is reduced in the dark-acclimated samples in many cyanobacterial species so that conventional protocol developed for land plants cannot be directly applied for cyanobacteria. Even for the estimation of the simplest chlorophyll fluorescence parameter, F_v/F_m, some additional protocol such as addition of DCMU or illumination of weak blue light is necessary. In this review, those problems in the measurements of chlorophyll fluorescence in cyanobacteria are introduced, and solutions to those problems are given.

Keywords Chlorophyll fluorescence · Cyanobacteria · Nonphotochemical quenching · Pulse-amplitude modulation (PAM) fluorometry · State transition

Abbreviations

DCMU	3-(3, 4-Dichlorophenyl)-1, 1-dimethylurea
F_m	Maximum fluorescence determined under oxidized plastoquinone pool conditions
$F_{m'}$	Maximum fluorescence under reduced plastoquinone pool conditions
F_0	Minimum fluorescence under oxidized plastoquinone pool conditions
$F_{0'}$	Minimum fluorescence under reduced plastoquinone pool conditions
F_s	Stable fluorescence level
F_v/F_m	Chlorophyll fluorescence parameter indicating the maximum quantum yield of Photosystem II calculated as $(F_m - F_0)/F_m$
NDH	NAD(P)H dehydrogenase
OCP	Orange carotenoid protein
PFD	Photon flux density
PQ	Plastoquinone
PS	Photosystem

Introduction

Chlorophyll fluorescence measurements have been widely used to estimate photosynthesis for several decades (Krause and Weiss 1984, 1991; Govindjee 1995). In particular, the emergence of commercially available pulse-amplitude modulation (PAM) fluorometer (Schreiber et al. 1986) prompted many people to use this technique for photosynthesis research. Because of its nondestructive nature of the PAM system, the technique has been used not only in laboratory measurements but also in field and ocean experiments. Simple comparison of the two levels of chlorophyll fluorescence of a dark-acclimated leaf, F_0 (the minimal fluorescence level) and F_m (the maximum fluorescence level of the same leaf upon saturating light), gives the most popular fluorescence parameter F_v/F_m, calculated as $(F_m - F_0)/F_m$. F_v/F_m theoretically represents the maximum quantum yield of Photosystem (PS) II (Kitajima and Butler 1975) and has been used almost as a synonym of PSII activity in wide ranges of papers.

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Since the yield of chlorophyll fluorescence from PSI is far lower than that from PSII at room temperature (Krause and Weis 1984), the change in the fluorescence level primarily reflects the change in the condition of PSII. One of the factors that affect the yield of chlorophyll fluorescence is the redox state of Q_A, an electron acceptor of PSII: The more Q_A is reduced, the higher the yield of fluorescence is. However, the change in the activities of the downstream components of PSII (e.g., cytochrome b_6/f and PSI complexes) may also affect chlorophyll fluorescence through the change in the redox state of plastoquinone (PQ) pool that is in equilibrium with Q_A in PSII. Thus, Φ_{PSII}, a chlorophyll fluorescence parameter that represents effective quantum yield of electron transport through PSII, reflects the yield of whole chain electron transport. The damage to PSI would lead to the decrease in Φ_{PSII} even if PSII complex is totally intact. Many other parameters were also invented to monitor different aspects of photosynthesis that are not restricted to the yield of PSII electron transport.

Many of these parameters are automatically calculated by measuring software upon determination of chlorophyll fluorescence, and such calculation usually assumes chlorophyll fluorescence characteristics of land plants. Eukaryotic algae and cyanobacteria are known to show different characteristics of chlorophyll fluorescence from those of land plants (Schreiber et al. 1995; Campbell and Öquist 1996). Accordingly, automatic calculation of chlorophyll fluorescence parameters without consideration of the characteristics of these organisms may result in meaningless values leading to wrong conclusion. Particularly, prokaryotic nature of cyanobacteria as well as their phycobilisome-based antenna systems complicates the determination of chlorophyll fluorescence (Campbell et al. 1998). It was reported that the results of chlorophyll fluorescence measurements did not reflect the condition of photosynthesis at all (Ogawa et al. 2013; Schuurmans et al. 2015; Ogawa and Sonoike 2016). In this review, we first take up the difference of the fluorescence characteristics between cyanobacteria and land plants, and then, problems raised by those differences and solution to those problems will be discussed in the following sections.

General characteristics of the quenching analysis in cyanobacteria compared with land plant leaves

Typical traces of the quenching analysis of chlorophyll fluorescence in land plants and cyanobacteria are shown in Fig. 1. First, let us look at the fluorescence level in the dark. In dark-acclimated plant leaves, the Q_A electron acceptor in PSII is fully oxidized (i.e., photochemical quenching is maximum) and no energy dissipation mechanism is induced (i.e., nonphotochemical quenching is minimum), giving the F_0 level in quenching analysis of chlorophyll fluorescence. Upon application of saturating pulse, fluorescence level increases to F_m, enabling us to calculate F_v/F_m (Fig. 1A-a). The values of healthy plant leaves are generally between 0.80 and 0.83, which are known to be well conserved among many plant species. In cyanobacterial cells, however, F_m level relative to F_0 level is much smaller than the case of land plant leaves (Fig. 1B-a), giving the apparent F_v/F_m values around 0.4–0.6 (Misumi et al. 2016). Cause of the lower values of the apparent F_v/F_m is not simple, and several factors are involved in this phenomenon as discussed in the following sections.

Next, photosynthesis under light condition is usually examined by the application of actinic illumination. After light acclimation of land plant leaves under certain actinic light condition, the level of fluorescence upon saturating pulse decreases to $F_{m'}$ level from the original F_m level in the dark, primarily due to the induction of energy

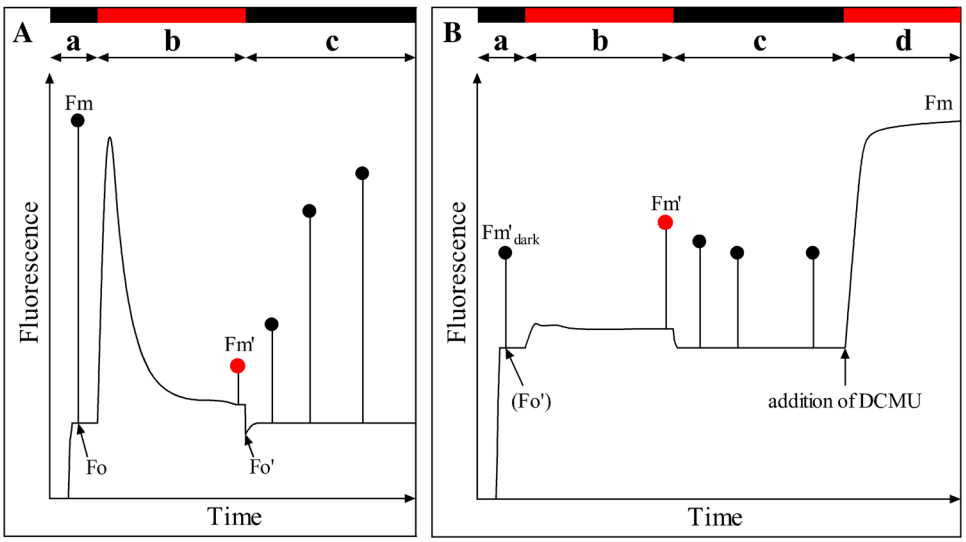

Fig. 1 Typical traces of chlorophyll fluorescence quenching analysis in land plants (**A**) and cyanobacteria (**B**). Measurements are conducted in four phases: dark-acclimated phase (*a*), light-acclimated phase (*b*), dark-recovery phase (*c*), and DCMU phase (*d*)

dissipation mechanism (i.e., increase in nonphotochemical quenching) (Fig. 1A-b). The fluorescence level itself transiently increases after onset of the actinic light but usually returns to the level near the original F_0 level observed in the dark, presumably due to the balance between the increase in nonphotochemical quenching and partial reduction of Q_A (i.e., decrease in photochemical quenching). During the course of light acclimation of cyanobacteria, however, $F_{m'}$ increases, not decreases (Fig. 1B-b). Evidently, the apparent F_m value in the dark-acclimated cells is not the true maximum level of chlorophyll fluorescence in many of the cyanobacterial species. To obtain the true F_m level, it is necessary to determine the fluorescence level in the presence of DCMU under light condition (Fig. 1B-d). Not only the level of fluorescence but also the kinetics of fluorescence is different in cyanobacteria compared with land plants. The fluorescence level increases after the onset of the actinic light and keeps the higher level than the F_0 level in the dark (Fig. 1B-b). In other words, increase of photochemical quenching is larger than the decrease of nonphotochemical quenching. The results suggest that the regulatory mechanism of the light acclimation system would be also different between cyanobacteria and land plants (Misumi et al. 2016).

Finally, let's look at the changes in the fluorescence level upon the cessation of the actinic light. In the case of land plant leaves, the fluorescence level would sharply decrease to the $F_{0'}$ level that is lower than the F_0 level in the dark, presumably due to the rapid oxidation of Q_A (i.e., increase of photochemical quenching) with the persistence of the nonphotochemical quenching (Fig. 1A-c). The fluorescence level gradually increases from $F_{0'}$ level to the original F_0 level through the relaxation of the nonphotochemical quenching in the subsequent dark period. In addition to this change, the transient increase of chlorophyll fluorescence due to cyclic electron transport might be observed. This transient increase has been widely used for the detection of cyclic electron transport mediated by NDH-1 complex (Munekage et al. 2004), and the increase is also observed in cyanobacteria. In the case of cyanobacteria, however, decrease of the fluorescence to the level lower than the original level in the dark is never observed (Fig. 1B-c). Thus, it is impossible to determine $F_{0'}$ level of cyanobacteria by looking at the post actinic kinetics of the fluorescence.

Due to these differences of chlorophyll fluorescence kinetics between land plants and cyanobacteria, it is difficult to estimate the condition of photosynthesis in cyanobacteria using standard analytical protocol developed for land plants. There are many problems in the chlorophyll fluorescence measurements in cyanobacteria that should be solved for the precise understanding of the cyanobacterial photosynthesis. In the following sections, the problems in the measurements in cyanobacteria and their solutions are discussed.

The mechanism of chlorophyll fluorescence quenching in cyanobacteria

As stated in the "Introduction" section, the level of chlorophyll fluorescence is affected by the redox state of Q_A. The mechanism of this type of quenching, the so-called photochemical quenching, is assumed to be the same between land plants and cyanobacteria based on the highly conserved reaction center complexes among oxygenic photosynthetic organisms. On the other hand, the mechanism of nonphotochemical quenching varies in different organisms reflecting the diversity of antenna systems of photosynthetic organisms. Nonphotochemical quenching in land plants is usually induced by relatively high actinic light, and relaxes in the subsequent darkness. This process is reflected in the change in $F_{m'}$ level, which is low under actinic light and becomes progressively higher during subsequent dark period (Fig. 1A-c). The recovery process consists of multiple phases, and Quick and Stitt (1989) attributed fast phase to energy-dependent quenching, middle phase to state transition, and slow phase to photoinhibition. With healthy plant leaves under physiological photon flux densities (PFD), most of the photochemical quenching is accounted for energy-dependent quenching mainly associated with xanthophyll cycle-type energy dissipation induced by the formation of proton gradient across thylakoid membranes. Cyanobacteria, on the other hand, do not have xanthophyll cycle. Campbell and Öquist (1996) demonstrated that the cyanobacterial nonphotochemical quenching was not affected by the addition of an uncoupler and did not show any correlation with ATP/ADP ratio in the cells. They concluded that main component of the cyanobacterial nonphotochemical quenching is state transition, not energy-dependent quenching.

State transition is the change in energy allocation between the two photosystems (Mullineaux and Emlyn-Jones 2005). In case of cyanobacteria, the movement of antenna phycobilisome from PSII to PSI results in more energy allocation to PSI (Mullineaux et al. 1997). In State 1, energy is more allocated to PSII, while energy is directed to PSI after transition to State 2. Since PSI has far lower fluorescence yield at room temperature than PSII, overall apparent fluorescence yield becomes smaller after the transition to State 2 from State 1. It must be noted that the decrease in fluorescence upon state transition is ascribed to the decrease in absorption cross section of PSII and not to the change in fluorescence yield within PSII core complex. Thus, at least from the PSII point of view, the word, "quenching," might be somewhat misleading to describe

the decrease of fluorescence due to state transition. However, if we think the two photosystems as one single system, the yield of fluorescence from PSI and PSII as a whole certainly decreases. In this review, we generally use the word, "quenching," irrespective of the mechanisms of the decrease of fluorescence. Only when the quenching of chlorophyll fluorescence is solely attributed to state transition, we use "state transition" instead of general "nonphotochemical quenching."

In addition to state transition, orange carotenoid protein (OCP) contributes to the decrease of chlorophyll fluorescence level. OCP is a small carotenoid protein that regulates the energy transfer from phycobilisome to PSII (Kirilovsky 2007). Upon relatively strong blue light illumination, OCP becomes active to suppress the energy transfer from phycobilisome to PSII. When red LED is used as an actinic light source as in many of the conventional PAM fluorometers, the OCP-induced fluorescence quenching is not observed. Under blue or white light excitation, however, energy dissipation by OCP is manifested as a form of nonphotochemical quenching. Actual quantitative estimation of OCP function is not so simple in cyanobacteria, since blue light also acts as PSI-excitation light in phycobilisome-containing organisms (see "The problems arise from pigment composition" section). In case of some marine cyanobacteria such as *Prochlorococcus*, OCP gene is absent in their genomes. Interestingly, however, strong energy-dependent-type fluorescence quenching is observed in *Prochlorococcus* (Bailey et al. 2005; Kühl et al. 2005) so that there might be some heat dissipation system of unknown mechanism. In any events, strong illumination of cyanobacterial cells could cause fluorescence quenching that is independent of state transition.

The problems arise from metabolic interaction

Cyanobacteria are prokaryotes that have no organelle to compartment cellular materials. Photosynthetic machinery is not enclosed in chloroplasts, and photosynthetic metabolism can interact all of the other metabolic pathways within the cells. Thus, the change in chlorophyll fluorescence that reflects the condition of photosynthesis might also reflect other metabolic pathways in cyanobacteria at least in theory. Actually, simple time series of chlorophyll fluorescence levels during illumination of dark-acclimated samples, so-called Kautsky curve, were shown to be affected by the disruptions of more than half of the genes in the genomes in *Synechocystis* sp. PCC 6803 (Ozaki et al. 2007). Since the disruption of genes in the same category, e.g., respiration, induced similar change in the Kautsky curve (Ozaki and Sonoike 2009), the changes in the chlorophyll fluorescence by the disruption of genes must be induced by the specific interaction between photosynthesis and other metabolic pathways.

In this context, the most direct interaction is observed between photosynthesis and respiration. Cyanobacterial respiratory electron transport uses PQ instead of ubiquinone, and both respiratory and photosynthetic electron transports share the same PQ pool in thylakoid membranes (Aoki and Katoh 1982; Peschek and Schmetterer 1982). Cytochrome b_6/f complexes and cytochrome c_6 (or plastocyanin) are also shared by the two electron transport chains (Fig. 2). Thus, the condition of respiratory electron flow is expected to affect chlorophyll fluorescence directly, and that is the case. In the darkness without photosynthesis, the redox state of the PQ pool is fully oxidized and cells are in State 1 in land plants. On the other hand, the redox state of the PQ pool in the dark-acclimated cyanobacterial cells should be determined by the respiratory activity, presumably by the balance between the electron input through NAD(P)H dehydrogenase complex (NDH) 1 [Respiration (inflow) in Fig. 2] and the electron output through oxidases [Respiration (outflow) in Fig. 2]. In case of many cyanobacterial species, $F_{m'}$ level is already quenched in the dark (Fig. 1B-a), and that could be explained by the transition to State 2 through the reduction of PQ pool, and that, in turn, might be explained by the fast electron flow from NDH-1 complex in the dark-acclimated cells (Mullineaux and Allen 1986).

Involvement of NDH-1 complex in the induction of State 2 in the dark-acclimated cyanobacterial cells is directly shown by the examination of the NDH-1 mutants in *Synechocystis* sp. PCC 6803. Lack of NDH-1 activity in the *ndhF1* disruptant or the *ndhB* disruptant (M55 mutant) resulted in the oxidation of PQ pool in the dark

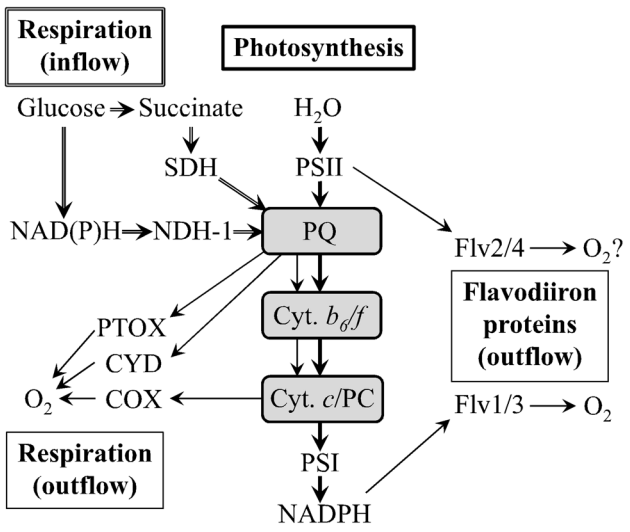

Fig. 2 Schematic model of interaction between photosynthesis and alternative electron transports in cyanobacteria

and the cells are locked in State 1 (Schreiber et al. 1995; Ogawa et al. 2013). Phenotype of the disruptant of *ndhF1* gene cannot be ascribed to the inability of state transition, since addition of KCN, an inhibitor of terminal oxidase, to the disruptant induced the transition to State 2 in the dark. Furthermore, the phenotype is specific to the disruption of the subunit involved in respiratory function: the disruption of other subunits of the NDH-1 complex, e.g., NdhF3 involving carbon-concentrating mechanism (Battchikova et al. 2011), resulted in totally different phenotypes (Ogawa and Sonoike 2015). Succinate dehydrogenase complex (SDH) was also reported to significantly affect the redox state of PQ pool (Cooley and Vermaas 2001). Since the inactivation of NDH-1 complexes may affect the supply of succinate through the lack of $NADP^+$ in tricarboxylic acid (TCA) cycle, interpretation of the change in metabolic interaction is not so simple.

The role of terminal oxidases encoded by *cox* or *cyd* genes is also important for the redox control of photosynthetic electron transport, since these genes were reported to be essential for the survival of *Synechocystis* sp. PCC 6803 upon rapid environmental shift from dark to high light condition (Lea-Smith et al. 2013; Ermakova et al. 2016). Inactivation of the *cyd* gene was reported to lead to the decrease in the effective quantum yield of PSII only under low light condition (Ermakova et al. 2016). Similarly, in a marine cyanobacterium *Synechococcus* WH8102, inhibition of plastoquinol terminal oxidase (PTOX) by propyl gallate was reported to lead to the reduction of PQ pool (Bailey et al. 2008). These results clearly indicate that state transition in cyanobacteria is under the control of the redox state of PQ pool that might be largely influenced by the activity of respiratory NDH-1 and/or terminal oxidases.

PQ pool reduction in the dark-acclimated cyanobacterial cells causes several problems. First of all, F_m level cannot be determined by the application of a saturating pulse on dark-acclimated samples as in land plant leaves, since the fluorescence level is quenched to $F_{m'}$ level due to state transition in the dark in cyanobacteria. To obtain true F_m level, illumination in the presence of DCMU is necessary (Campbell et al. 1998). Addition of DCMU blocks electron transfer from Q_A to Q_B, resulting in the oxidation of PQ pool that brings the cells to State 1 with low fluorescence quenching. Since many photosynthetic parameters (e.g., F_v/F_m and NPQ) require the F_m level for calculation and most software attached to fluorometer assumes the maximum fluorescence level in dark-acclimated samples as F_m level, calculated parameters in the measurements of cyanobacterial cells are sometimes nothing but a nonsense. Manual calculation using the F_m level obtained by the addition of DCMU is necessary for the measurements of cyanobacterial cells. Alternatively, weak blue light illumination can be used for the determination of the F_m level as described in the next section.

Secondly, the chlorophyll fluorescence parameter Φ_{PSII} is not reliable to estimate electron transport efficiency in cyanobacteria. Φ_{PSII} represents quantum yield of photosynthetic electron transport through PSII and is widely used for its simple calculation procedure: Φ_{PSII} can be calculated as $(F_{m'}-F_s)/F_{m'}$, which only requires the levels of stable fluorescence, F_s, together with $F_{m'}$ that can be determined at any point without any need of reference points such as F_m or F_0 for the calculation. That enables us to record Φ_{PSII} continuously, e.g., during culturing of cells. However, the above-mentioned *ndhF1* disruptant showed higher Φ_{PSII} than wild-type cells irrespective of its lower oxygen-evolving activity (Ogawa et al. 2013; Fig. 3). The discrepancy could be at least partly ascribed to the apparent lower yield of photosynthesis in wild-type cells than in the *ndhF1* disruptant due to state transition. In dark-acclimated cells, the wild-type cells are in State 2 so that the absorbed light energy is more allocated to PSI resulting in apparent higher energy dissipation as a whole photosynthetic machinery. Upon weak light illumination, photosynthetic electron transport kicks into action, and PQ pool becomes gradually oxidized depending on the balance between PSI and PSII activities. Even in the light, however, the PQ pool in the wild-type cells is tended to be more reduced than that in the *ndhF1* disruptant, resulting in the lower Φ_{PSII} in the

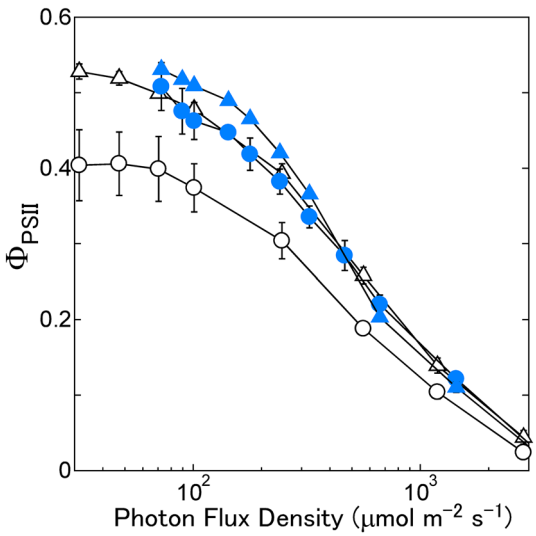

Fig. 3 Actinic light dependence of Φ_{PSII} in the wild-type cells (*circles*) or the *ndhF1* disruptant (*triangles*) of *Synechocystis* sp. PCC 6803. Cells are dark-acclimated for 10 min, and measured with red actinic light (660 nm). *Open symbols* no other background light. *Closed symbols* cells are illuminated by background weak blue light (460 nm, 70 μmol m^{-2} s^{-1}) during dark-acclimation as well as during the measurement. Data of open symbols are taken from Ogawa et al. (2013)

wild-type cells. The situation is also similar in other "yield type" parameters such as F_v/F_m or $F_v/F_{m'}$.

Thirdly, the level of true F_0 is also not so easy to determine in cyanobacteria. As discussed extensively above, PQ pool is reduced in the dark and the cells are in State 2 in many cyanobacterial species. Thus, the original fluorescence level of the dark-acclimated cells should be regarded as quenched $F_{0'}$ level, not F_0 level (Ogawa and Sonoike 2016). The cells must be brought to State 1 in order to determine true F_0 level just in the case of F_m measurements described above. Illumination in the presence of DCMU, the condition to determine true F_m, cannot be used for the determination of true F_0, since the condition also reduces Q_A while F_0 determination requires oxidized Q_A. One effective way to oxidize both PQ pool and Q_A is blue light illumination. As will be discussed in the next section, appropriate blue light acts as PSI-excitation light and oxidizes PQ pool as well as Q_A in many cyanobacteria (Schreiber et al. 1995). Actually, under background illumination of weak blue light, the difference of Φ_{PSII} between the wild-type cells and the *ndhF1* disruptant described above is largely diminished (Fig. 3), presumably because of the oxidation of PQ pool by the blue light illumination. Similarly, PQ pool that has been reduced in the dark becomes oxidized by blue light illumination, manifested by the increase of fluorescence level from quenched $F_{0'}$ to true F_0. The true level of F_m can be also determined with saturating pulse under blue light in the absence of DCMU (e.g., El Bissati et al. 2000). The blue light illumination should be weak enough to avoid the induction of OCP-dependent quenching of chlorophyll fluorescence but strong enough to oxidize PQ pool completely.

Dark-induced State 2 is also reported in marine cyanobacteria such as *Synechococcus* sp. WH 8102 (Bailey et al. 2005) or *Synechococcus* sp. WH 5701 (Kaňa et al. 2012) as well as in freshwater cyanobacteria. Generally speaking, chlorophyll fluorescence characteristics of marine cyanobacteria are not especially different from those of freshwater cyanobacteria, although wide variation was observed within each group. Very peculiar pigment composition of some marine cyanobacteria does not seem to interfere the chlorophyll fluorescence measurements considerably, provided that the photosynthetic pigments are properly excited by appropriate color of light (see next section). It must be noted, however, that the oxidized PQ pool in dark-acclimated cells is observed in some cyanobacterial species, giving similar fluorescence characteristics to those of land plants. For example, PQ pool of *Anabaena* sp. PCC 7120, *Nostoc* sp. HK-01, *Arthrospira platensis* NIES-39, and *Synechocystis* sp. PCC 6803 was reduced in the dark while that of *Acaryochloris marina* MBIC 11017 and *Nostoc punctiforme* ATCC 29133 was more or less oxidized in the dark (Misumi et al. 2016). Since two species in the same genus *Nostoc* showed totally different redox states of PQ pool, the difference is not brought about by the phylogenetic difference. There seems to be a tendency that the stronger the light of original habitat of the species is, the more reduced the PQ pool in the dark is. Such relationship would be a suggestive fact to consider the physiological relevance of the reduced PQ pool in the dark.

Other than respiratory electron transport, oxygen reduction on the PSI acceptor side through flavodiiron (Flv) proteins also affects the redox state of photosynthetic electron transport (Fig. 2). PSI is susceptible to photoinhibition when electron acceptors are reduced (Sonoike 2011), so that the oxidation of electron acceptor through Flv works as a protective mechanism for PSI against photoinhibition (Allahverdiyeva et al. 2013). Since alternative electron flow to oxygen results in the apparent consumption of oxygen, the photosynthetic activity estimated by the rate of oxygen evolution and that estimated by chlorophyll fluorescence measurement would not match in the presence of Flv function (e.g., Hayashi et al. 2014). In other words, difference of the activities between two methods can be ascribed to the presence of alternative electron flow to oxygen. The decreased activity of oxygen evolution in the *ndhF1* disruptant discussed above might be also explained by the enhanced oxygen consumption through the induction of Flv proteins in the disruptant. Seemingly, chlorophyll fluorescence measurements are more reliable than oxygen evolution measurements in the presence of alternative electron flow to oxygen. Although the defects in Flv1/3 (Hayashi et al. 2014) as well as those in Flv2/4 (Zhang et al. 2009; Shimakawa et al. 2015) certainly affect the condition of photosynthetic electron transport, it seems that the observed changes in chlorophyll fluorescence more or less reflects the true changes in photosynthesis. In that sense, the defects in alternative electron transfer to oxygen interfere only photosynthesis, and do not interfere the measurement of chlorophyll fluorescence. The smaller effect of Flv inactivation on chlorophyll fluorescence measurements compared with respiratory NDH inactivation could be ascribed to the fact that respiration mainly affects the redox state in the dark where photosynthesis is not active, while Flv works under the light condition together with PSI. Thus, we should be especially cautious to interpret the differences in chlorophyll fluorescence parameters determined in the dark or under low light in the analysis of cyanobacteria.

The problems arise from pigment composition

In the case of land plants, the number of chlorophylls serving for PSI is generally similar to that of chlorophylls serving for PSII. On the other hand, the number of PSI

chlorophyll is five times to ten times larger than that of PSII chlorophylls in cyanobacteria (Campbell et al. 1998) presumably because of the presence of phycobilisome that serves as antenna primarily for PSII. Chlorophylls mainly absorbs blue light around 400–480 nm and red light around 650–700 nm, while phycobilisome mainly absorbs yellow/orange light around 500–680 nm. Taken together, blue light solely absorbed by chlorophyll would act as PSI-excitation light, while yellow/orange light below 650 nm would act as PSII-excitation light in many cyanobacterial species.

There are pros and cons in this feature of cyanobacteria. The wavelength separation for PSI-excitation light and PSII-excitation light in phycobilisome-containing organisms is very distinct, compared with the case of land plants where chlorophylls are served for both PSI and PSII antenna systems. It is necessary to use far red light above 700 nm that is absorbed in long-wavelength chlorophylls in PSI in order to excite land plant PSI, selectively. This is also true for some cyanobacteria with low or no phycobilisome, e.g., some marine species such as *Prochlorococcus*. Furthermore, a marine cyanobacterium *Synechococcus* WH8102 shows lower PSI/PSII ratio than typical fresh water cyanobacteria (Bailey et al. 2008) so that selective excitation of PSI is also not so simple. On the other hand, simple illumination of weak blue light can be used for PSI excitation in many cyanobacteria with high PSI/PSII ratio and using phycobilisome as main pigment. Overall excitation yield by blue light in such cyanobacteria, however, is very low so that, if blue light is used for saturating pulse as in some old PAM instruments, the pulse may not be saturating for many cyanobacteria. Furthermore, some types of fluorometers use blue light also for measuring light in default. In that case, the sensitivity would be very low for such cyanobacteria but the problem can be overcome by replacing the blue light source by orange one. Alternatively, the introduction of multicolor measuring light to chlorophyll fluorometer that has recently become more familiar to photosynthesis community is very useful for the fluorescence measurements of organisms with different pigment compositions.

Attention should be also paid for the determination of OCP function, since blue light illumination induced both energy dissipations by OCP systems and PQ pool oxidation by PSI excitation. To separate the two phenomena, the difference between weak blue light and strong blue light should be examined, since weak blue light is known to induce oxidation of PQ pool but does not induce OCP function (Kirilovsky 2007). It must be also noted that, due to the good separation of the wavelengths of light for PSI and PSII excitation, even "white" light might be act as either PSI-excitation light or PSII-excitation light, depending on the characteristics of the light source. During the quenching analysis of chlorophyll fluorescence of cyanobacteria, some overshoot might be observed after application of a saturating pulse: the fluorescence level goes up to $F_{m'}$ level upon application of the saturating pulse under continuous actinic light, and decreases to the level lower than the original level before the saturating pulse, and then gradually goes back to the original level again. Several causes could be considered for this type of overshoot change including the transient preamplifier saturation in electric circuit but one possibility is the induction of state transition during the pulse. If the actinic light source has spectral feature to excite PSII relative to PSI and the spectra of the saturating pulse are in reverse, the application of saturating pulse induces rapid state transition to State 1 to State 2 to decrease the fluorescence level that can persist some while after the saturating pulse. In cyanobacteria, one should pay more attention to the spectral feature of the light source compared with the case of land plants.

Cyanobacteria do not have land plant type LHC complexes containing chlorophyll *b* so that cyanobacterial photosynthetic pigments are categorized to three groups: PSI core (chlorophyll *a*), PSII core (chlorophyll *a*), and phycobilisome (e.g., allophycocyanin, phycocyanin, and phycoerythrin). Since fluorescence emission from either PSI or from phycobilisome contributes to F_0 but does not contribute to variable fluorescence, $F_v = F_m - F_0$, the change in the relative ratio of three pigment groups is expected to affect F_v/F_m value. In the case of land plants that have no phycobilisome, the ratio of PSI to PSII is the factor that affects F_v/F_m (Genty et al. 1990; Pfündel 1998). Pfündel et al. developed the method to estimate the PSI contribution to F_0 level (Pfündel et al. 2013). This method is based on the theoretical calculation of $F_{0'}$ level introduced by Oxborough and Baker (1997). The calculation of $F_{0'}$ is based on the assumption that the quenching ratio of F_m to $F_{m'}$ and that of F_0 to $F_{0'}$ is the same. However, the calculated $F_{0'}$ would be different from the actual $F_{0'}$ in the presence of PSI fluorescence, since the fluorescence from PSI, which does not quench as that from PSII, contributes to F_0 but contributes less to F_m. In other words, the comparison of the actual $F_{0'}$ level and calculated $F_{0'}$ level gives information about the relative contribution from PSI fluorescence. Through this type of analysis, Pfündel et al. (2013) successfully estimate the contribution of PSI fluorescence in C_3 and C_4 plants.

Similar estimation of "basal" fluorescence from PSI and phycobilisome would be extremely important and theoretically possible in cyanobacteria. Since many cyanobacteria have highly fluorescent phycobilisome and high PSI/PSII ratio (Campbell et al. 1998; El Bissati and Kirilovsky 2001), the contribution of "basal" fluorescence in cyanobacteria should be far higher than in land plants. It must be noted that high fluorescence from phycobiliproteins is observed not only in phycobilisome energetically decoupled from photosystems but also in well-coupled one

(Mimuro and Kikuchi 2003). Phycobiliproteins have higher intrinsic fluorescence yield than that of chlorophylls. As mentioned in the previous section, however, the actual determination of F_0 and $F_{0'}$ in cyanobacteria would be totally different from that in land plants. Taking these factors into account, the contribution of "basal" fluorescence from PSI and phycobilisome was estimated in *Synechocystis* sp. PCC 6803, and "true" F_v/F_m was calculated in the absence of the "basal" fluorescence (Ogawa and Sonoike 2016). The obtained value of "true" F_v/F_m is around 0.8, which is much higher than the "apparent" F_v/F_m in many cyanobacterial species (Misumi et al. 2016) and more similar to the value observed in land plants. Similar compensation for the phycobilisome fluorescence was also conducted by calculation using fluorescence yield of each photosynthetic pigment, although the effect of state transition was not considered in the study (Acuña et al. 2016). The results indicate that the maximum photochemical efficiency of PSII is similar between land plants and cyanobacteria, which appears to be reasonable considering the highly conserved structure of PSII reaction center in land plants and cyanobacteria.

This type of correction for the "basal" fluorescence is quite important to compare the two samples with different pigment compositions. When cyanobacteria grow under nutrient deficient condition, the drastic decrease of phycobilisome content, the so-called bleaching is often observed (Allen and Smith 1969; Grossman et al. 1993). Typical bleaching is observed by the nitrogen starvation or sulfur starvation, and color of the cell culture turns from blue-green to yellow. From the determination of oxygen evolution, the decrease in the yield of PSII under nitrogen-deficient condition was proposed (Li and Sherman 2002). This proposal could not be supported by the chlorophyll fluorescence determination in the presence of "basal" fluorescence, but subtraction of the "basal" fluorescence clearly indicates the decrease of "true" F_v/F_m under nitrogen starvation (Ogawa and Sonoike 2016). It must be noted, however, that this type of correction by subtraction often leads to the large variance of the corrected value. The problem arises especially when cells with low phycobilisome content is used, since the decrease of phycobilisome would usually lead to the poor excitation of PSII and low sensitivity in the fluorescence measurements. Data with small errors should be used for the correction of the "basal" fluorescence.

It must be noted that the level of the "basal" fluorescence is largely dependent on the color of measuring light. Although the "basal" fluorescence from phycobilisome is significant in orange/red light excitation, it is negligible in the excitation at chlorophyll absorption maximum around 435 nm due to low absorption of phycobilisome at this region. On the other hand, the "basal" fluorescence from PSI would persist either in orange/red light excitation or in blue light excitation. Thus, the determination of the parameters such as F_v/F_m in phycobilisome-containing organisms should be much more accurate in blue light excitation, if the above-mentioned problem due to low fluorescence yield can be solved. Since some marine cyanobacteria, e.g., *Synechococcus* WH8102, contain phycourobilin that absorbs shorter wavelength light compared with other phycobilins (Blot et al. 2009), some blue light sources provided by the conventional chlorophyll fluorometers may not be appropriate for the purpose of avoiding fluorescence from phycobilin in such species. Here again, the introduction of multicolor measuring light to fluorescence measurements is very useful for the accurate determination of chlorophyll fluorescence parameters of organisms with different pigment compositions.

Other problems

In this section, we would like to briefly summarize several other problems in cyanobacterial chlorophyll fluorescence measurements that have been discussed in the past literatures. First of all, the rate of development of nonphotochemical quenching in cyanobacteria is much higher than that in land plants (Schreiber et al. 1995). Fluorescence is quenched within the order of 10 ms, even during the saturating pulses. State transition is really a quick process in cyanobacteria. This makes the determination of F_m or $F_{m'}$ in cyanobacteria by old-fashioned chart recorder difficult but it is not problem at all in recent digital data handling system with fast time resolution. Secondly, automatic calculation of electron transport rate (ETR) might become problematic in cyanobacteria. In the default setting of many chlorophyll fluorescence instruments, ETR is calculated as $0.5 \times 0.84 \times \mathrm{PFD} \times \Phi_{\mathrm{PSII}}$, where PFD is the photon flux density of the actinic light. Since the first figure in the equation, 0.5, is based on the equal distribution of light energy between PSI and PSII, this is not applicable to cyanobacteria as discussed in the previous section. The second figure, 0.84, stands for the typical absorptance of plant leaves so that this is also not applicable to cyanobacterial measurements. When precise determination of energy distribution between two photosystems and absorptance of cell suspension is not possible, not ETR itself, but relative value of ETR (rETR calculated as $\mathrm{PFD} \times \Phi_{\mathrm{PSII}}$) should be used to compare the photosynthetic electron transport rate.

Finally, there is one problem that has not been solved until now. In cyanobacteria, state transition can be estimated as qN (or NPQ), the parameters representing nonphotochemical quenching (Campbell and Öquist 1996). The state transition is known to be regulated by the redox state of PQ pool (Mullineaux and Allen 1986) that can be

estimated as qP (or qL). Dark-acclimated cells are in State 2 so that qN is high but weak light around growth light level brings the cells to State 1, resulting in low qN. When light level becomes much higher, cells are brought back to State 2 and qN increased again. Such concave dependence of qN has been widely reported in the past (e.g., Campbell and Öquist 1996; Sonoike et al. 2001). Since PQ pool should be reduced in State 2, qP should be low in the dark and under high light while it should be high under growth light region. However, such convex dependence of qP on actinic light has never reported; the reported actinic light dependence of qP is usually monotonous (e.g., Campbell et al. 1998; Sonoike et al. 2001). The part of the discrepancy might be attributed to the quenching of fluorescence of dark-acclimated cells from F_0 to $F_{0'}$ as discussed in the "The problems arise from metabolic interaction" section. The parameter, qP, was calculated on the assumption that the original fluorescence level of dark-acclimated cyanobacterial cells was F_0, not $F_{0'}$ in many of the past studies (e.g., Ogawa et al. 2013). Some authors hypothesized that the discrepancy could be attributed to the nonequilibrium between PQ pool and Q_A in PSII (e.g., Campbell et al. 1998) but the hypothesis is yet to be proved. In the case of the regulation of photosystem stoichiometry in cyanobacteria, quinone-binding site of cytochrome b_6/f complex was once proposed as the monitoring site for the regulation (Fujita 1997). Considering that state transition in land plants is monitoring the redox state of cytochrome b_6/f complex (de Vitry et al. 2004), monitoring point for the regulation of state transition in cyanobacteria might be also some component in cytochrome b_6/f complex, which is in equilibrium with PQ pool. The mechanism of the redox control of the state transition in cyanobacteria may be using some special trick that should be clarified in future.

Conclusion

Although photosynthetic reaction center complexes are well conserved between land plants and cyanobacteria, standard method developed for the measurements of chlorophyll fluorescence of land plants cannot be used for cyanobacteria in many cases. The difficulties mainly arise from (1) reduced PQ pool in dark-acclimated cyanobacterial cells, (2) different pigment compositions in cyanobacteria, (3) large contribution of state transition in the absence of energy-dependent quenching, and (4) optical property of cell suspension different from that of plant leaves. Some of these difficulties could be avoided by the certain experimental protocol such as blue light illumination (for reduced PQ pool in the dark), while others could be compensated by the data processing (calculation of "basal" fluorescence from phycobilisome). The most important point in the chlorophyll fluorescence measurements is the correct measurements of two basic fluorescence values, F_m and F_0. In principle, these values can be determined in cyanobacteria under PQ-oxidizing conditions such as under blue light illumination or illumination in the presence of DCMU. Furthermore, the F_0 value should be corrected for the "basal" fluorescence from PBS and PSI by the equation originally developed for the correction of PSI fluorescence in land plants. It should be also noted that some of these difficulties are present in the experiments using eukaryotic algae too. At any event, it is important to recognize the existence of differences between land plants and cyanobacteria for appropriate measurements of chlorophyll fluorescence. It is not so difficult (although it may be a bit complicated) to appropriately estimate cyanobacterial photosynthesis by chlorophyll fluorescence measurements, but only after the true natures of the problems are made clear.

Acknowledgements This work was supported by JSPS Grant-in-Aid for Scientific Research on Innovative Areas (No. 16H06552 and No. 16H06553 to K.S.) and Grant-in-Aid for Scientific Research (B) (No. 16H04809 to K.S.), as well as by Grant-in-Aid for JSPS Research Fellow (No. 26-7221 to T.O.). We thank Dr. Yukako Hihara for the critical reading of the manuscript.

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