Technical Paper

The Principle of Fast Repetition Rate Fluorimetry

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1. Principle of Fast Repetition Rate (FRR) Fluorometry

1.1 Background

The yield of chlorophyll fluorescence in phytoplankton and higher plants is controlled by their ability to utilize light for photosynthesis. This ability varies due to environmental factors such as nutrient and trace metal availability, temperature, and exposure to high irradiance and/or UV content in the ambient light. It is also controlled by internal mechanisms of cell division and cell cycle. Finally, photochemical efficiency is strongly affected by short-term phenomena such as the actual level of photosynthetic activity under ambient irradiance, level of reduction of electron acceptors in Photosystem II (QA, QB, and the PQ pool), changes in the rates of energy desaturation within the light absorbing pigments, and variations in the rates of electron transport at terminal stages of carbon fixation. All these events modify the patterns of light utilization and the patterns of fluorescence response to the excitation light. Measurements and interpretation of these patterns provide information about the physiological performance of photosynthetic organisms, about the environmental factors controlling this performance, and the biophysical mechanisms of photosynthesis. As the fluorescence measurements are non-invasive and can be easily performed in situ, fluorescence techniques provide a convenient tool for characterization of photosynthetic apparatus in field conditions.

One of the most common approaches toward assessing the photosynthetic performance is to measure changes in the chlorophyll fluorescence in response to the excitation light. Fluorescence signals induced by actinic light are used extensively to derive photosynthetic parameters related to Photosystem II (Krause and Weis 1991). Measurement of the change in the quantum yield of fluorescence is non-destructive, non-invasive, and rapid, offers great sensitivity, and can be performed in real-time (Falkowski and Kolber 1995). Theoretical and empirical models have been proposed that relate the variations in fluorescence yields to the quantum efficiency of photochemistry (Butler and Kitajima 1975), (Weis and Berry 1990), the number of primary and secondary electron acceptors (Maatin and Kok 1965), (Murata, Nishimura et al 1966), the effective absorption cross section (Bonavia and Myers 1969), (Ley and Mauzerall 1982), (Falkowski, Wyman et al. 1986), (Falkowski and Kolber 1995), (Samson and Bruce 1995), energy transfer between PSII units (Pailletin 1976), (Ley and Mauzerall 1986), as well as the kinetics of electron transport between PSII and PSI (Bouges-Bocquet 1973), (Crofts, Baroli et al 1993), (Bowers and Crofts 1983). Changes in some or all of these parameters are frequently exploited not only to understand fundamental biophysical properties of the photosynthetic energy conversion, but also to examine how environmental stresses affect the function of PSII in vivo and to estimate photosynthetic electron transport under ambient irradiance (Kolber and Falkowski 1993).

The easiest and earliest technique for measuring changes in fluorescence yields is based on the analysis of a fluorescence transient induced in a dark adapted sample by rapid exposure to continuous light. The so-called fluorescence induction technique is intuitively simple; however,
because the rate of excitation delivery to PSI reaction centers is generally lower than the initial rate of $Q_{n}$ oxidation in a dark-adapted state, the observed fluorescence transient saturates only following the reduction of the plastoquinone pool. Hence, the kinetics of the changes in fluorescence yields are complicated by multiple photochemical turnovers. This complication can be avoided by the application of DCMU or similar herbicides that prevent reoxidation of $Q_{n}$. Unfortunately, this approach is destructive, cannot be used in real time, and in addition to blocking $Q_{n}$ reoxidation, may alter the normal pathways of electron transport and change distribution around PSI.

Hence, alternative fluorescence-based techniques have emerged and have proven to be extremely adaptable to real-time, non-destructive measurements. These techniques are based on the delivery of one or more pulses of saturating actinic light, such that fluorescence rises to a maximum as the acceptors in PSI become reduced. Two basic variations of this approach have been used extensively. The first is the pulse amplitude modulated (PAM) method (Shiels, Siliwa et al. 1986). This method uses a multiple turnover (MT) actinic flash to monitor the maximum fluorescence level in the presence of background irradiance. A MT flash is defined as a prolonged excitation that allows multiple photochemical electron transport. The second approach, based on a pump-and-probe (P&P) technique (Mauzerall 1972), (Falowski, Wyman et al. 1986), (Falowski, Kolber et al. 1988), compares the change in fluorescence yield of a weak probe flash prior to and following a single turnover (ST) actinic pump flash. A ST flash is operationally defined as a short excitation pulse that induces a single photochemical electron transfer. By varying the intensity of the actinic flash, the P&P method allows derivation of the functional absorption cross section of PSI (Falowski, Wyman et al. 1986). By varying the time delay between the actinic flash and the probe flash(es), the kinetics of electron transfer on the acceptor side of PSI can be assessed (Crofts, Baroli et al. 1993). Although the PAM and P&P techniques are superior to the induction method, PAM approach is limited to measurements of variable fluorescence, while measurements of the functional absorption cross section and the kinetics of electron transport in P&P method are not rapid enough to follow dynamic changes in PSI photophysical parameters occurring on time scales of milliseconds to minutes.

Fast Repetition Rate (FRR) fluorometry was developed to overcome the problems associated with both the PAM and P&P fluorometry, to expand the range of measured photophysical parameters, and to measure them on time scales of microseconds to milliseconds.

1.2. The Theory of Fast Repetition Rate Fluorometry

The basis of the FRR technique is to measure fluorescence transients induced by a rapid train of subsaturating excitation "flashlets", where the intensity, duration, and time delay between flashlets can be individually controlled. With peak excitation energies of up to 0.03 mole quanta m$^{-2}$ s$^{-1}$, and programmable duration and time interval between flashlets, FRR techniques allows to selectively manipulate the reduction states of the $Q_{n}$ and PQ pools and to assess the fluorescence yield change due to selective reduction of these two acceptors. Moreover, if the rate of excitation absorption by PSI greatly exceeds the rate of $Q_{n}$ oxidation, the kinetics of the fluorescence
transient can be used to calculate the functional absorption cross section of PSII, as well as the extent of energy transfer between PSII reaction centers within a single photochemical turnover of PSII. We will discuss the theory of FRR operation using a simplified model of PSII as presented in Fig. 1.

Quanta of light generated during excitation protocol are absorbed by the light absorbing pigment, LHCII, and transferred, via chlorophyll excited states, to PSII reaction center, RCII. Molecular organization of the LHCII/RCII permits cooperative light collection, either by sharing a common pool (or a fraction thereof) of LHCII, or by exchanging excitation energy between reaction centers. The overall efficiency of light trapping and excitation transfer to the reaction center is described by the functional absorption cross section, \(\Phi_{o_{\text{PSII}}}\). Excitation energy arriving at open PSII reaction centers (the electron acceptor \(Q_{b}\) in oxidized state) initiates a photochemical act, resulting in reduction of \(Q_{a}\) and oxidation of the electron donor, \(P_{680}\), with relatively little energy dissipated as heat and fluorescence. As a result, the fluorescence yield in an open PSII reaction center is at minimal level, \(F_{o}\). Oxidized \(P_{680}\) is reduced within about 100 ns by an electron donor \(Z\), while reoxidation of \(Q_{a}\) proceeds via a series of successive electron transport to \(Q_{b}\), PQ pool, and PSI. In average, this latter process requires from 0.6 to 10 milliseconds, depending on

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**Figure 1. Simplified model of Photosystem II.**

(a) Reaction center open:
- Fluorescence: 2%
- Photochemistry: 65%
- Heat: 33%

(b) Reaction center closed:
- Fluorescence: 6%
- Photochemistry: 97%
- Heat: 94%
the level of photosynthetic activity and reduction level of PQ pool. During this time PSII reaction center remains closed (Q$_{A}$ in reduced state), and the next arriving excitation will either be dissipated in a process of fluorescence/thermal deactivation, or transferred to another PSII reaction center. As a result, the fluorescence yield in a closed PSII reaction center increases to a maximum level, F$_{m}$, and the observed fluorescence signal, F, changes from F$_{o}$ to F$_{m}$ in proportion to the fraction of closed reaction centers.

Interpretation of the fluorescence transients induced by FRR excitation protocols allows calculation of a range of photosynthetic parameters such as F$_{o}$ (minimal), F$_{m}$ (maximal) and F$_{v}$ (variable) components of PSII fluorescence, functional absorption cross section, $\alpha_{PSII}$, and the kinetics of electron transfer between Q$_{A}$ and PQ pool.

Following the model of PSII in Fig. 1., the stimulated fluorescence signal, f(t), observed during FRR excitation protocol, is determined by the excitation signal, i(t), the functional absorption cross section, $\alpha_{PSII}$ and the kinetics of Q$_{A}$ reoxidation. Formally, the fluorescence yield $\tilde{f}(t)$ observed during excitation protocol can be described as:

\[f(t) = F_o \cdot (F_m - F_v) \cdot C(t)\]  

where F$_{o}$ is the minimal fluorescence yield observed when all PSII reaction centers are open, F$_{m}$ is the maximum fluorescence yield observed when all PSII reaction centers are closed, and C(t) is the fraction of reaction centers that are closed at time t.

Two processes control C(t) during FRR excitation protocol, namely: the absorption of the excitation energy followed by primary photochemistry, and reoxidation of Q$_{A}$. By definition, functional absorption cross section describes the efficiency of light utilization for photosynthesis in open PSII reaction centers. Formally $\alpha_{PSII}$ is expressed as:

\[\alpha_{PSII} = \frac{C}{\frac{\partial C}{\partial t}} \bigg|_{C_{eq}} = -\frac{\partial q}{\partial t} \bigg|_{C_{eq}}\]  

where I is the cumulative excitation energy, and q = 1 - C is the photochemical quenching, or a measure of a fraction of open RCII. Changes in the fraction of closed RCII during FRR excitation protocol can therefore be described as:
\[ \frac{\partial C(t)}{\partial t(t)} = \frac{\partial C(t)}{\partial t} \frac{1}{i(t)} = \alpha_{\text{PSII}} \left(1 - C(t) \right). \]  

(3)

which in the absence of \(Q_{A}^*\) reoxidation simplifies to:

\[ \frac{dC(t)}{dt} = \alpha_{\text{PSII}} \left(1 - C(t) \right). \]  

(4)

Eqn. 4 can be expressed as:

\[ C(t) = \int_{0}^{t} \alpha_{\text{PSII}} \left(1 - C(u) \right) du. \]  

(5)

where \(u\) is the integration variable. Solving Eqn. (5) yields

\[ C(t) = 1 - \exp \left[ \int_{0}^{t} \alpha_{\text{PSII}} \left(u - C(u) \right) du \right]. \]  

(6)

Eqns. (4) to (6) are valid when the rate of excitation delivery to RCII, \(i_{\text{PSII}}\), greatly exceeds the rate of \(Q_{A}^*\) reoxidation, i.e. at very high excitation energies, or when \(Q_{A}^*\) reoxidation is inhibited (for instance by DCMU). To account for the effects of \(Q_{A}^*\) reoxidation in a general case, Eqn (6) is modified to:

\[ C(t) = \int_{0}^{t} \alpha_{\text{PSII}} \left(1 - C(u) \right) g(u, t-u) du. \]  

(7)

where \(g(u, t-u)\) describes the extent of \(Q_{A}^*\) reoxidation in these reaction centers that were closed at time \(u\) during the course of excitation protocol, and reopened in the time interval \(t\) - \(u\). This function can be expressed as a sum of exponential components:

\[ g(\Delta t) = \alpha_{1} \exp \left(-\Delta t / \tau_{1} \right) + \alpha_{2} \exp \left(-\Delta t / \tau_{2} \right) + \alpha_{3} \exp \left(-\Delta t / \tau_{3} \right). \]  

(8)
All the parameters \( F_o, F_n, \sigma_{psb}, \) and the kinetic constants in Eqn. (8) can be calculated by numerically fitting the experimental fluorescence transient to Eqn. (1).

Eqn. (5) can be expressed in terms of fluorescence signal \( F_o, F_n \) and \( R(t) \):

\[
C(t) = \sigma_{psb} \int_0^t \frac{F_o - R(u)}{F_o - F_n} du
\]

(9)

Assuming \( C(t=\infty) = 1 \), Eqn. (9) allows calculation of \( \sigma_{psb} \) as:

\[
\sigma_{psb} = \left[ \int_0^\infty \frac{F_o - R(t)}{F_o - F_n} dt \right]^{-1}
\]

(10)

Estimate of \( \sigma_{psb} \) as described by Eqn. (10) is used as an initial guess in an iterative fitting procedure employing the exact solution given by Eqn. (7).

Maximum quantum yield of photosynthesis, \( \Phi_{o}^{\text{max}} \), is defined as a ratio of primary photochemistry to the light absorbed by PSII unit in open PSII reaction centers:

\[
\Phi_{o}^{\text{max}} = \frac{\partial C}{\partial(\sigma_{psb})/\sigma_{psb}} \bigg|_{C=0} = \frac{1}{\sigma_{psb}} \frac{\partial C}{\partial \sigma_{psb}} \bigg|_{C=0}
\]

(11)

where \( \sigma_{psb} \) is the optical absorption cross section of PSII unit. Assuming that portion of the absorbed light is used for photochemistry, with the remainder dissipated as heat and fluorescence (Fig. 1), \( \Phi_{o}^{\text{max}} \) can also be expressed in terms of photochemical, thermal, and fluorescence rates, \( k_p, k_o, \) and \( k_f \) respectively (Butler 1972), (Butler 1977):

\[
\Phi_{o}^{\text{max}} = \frac{k_f}{k_p + k_o + k_f}
\]

(12)

Similarly, the quantum yield of \( F_o \) fluorescence, \( \Phi_{o}^{\text{x}} \), observed in open PSII reaction centers (where photochemistry operates at rate \( k_p \)) can be expressed as:
\[ \Phi'_r = \frac{k_r}{k_r + k_b + k_I} \]  

(13)

and the quantum yield of \( F_o \) fluorescence, \( \Phi'_o \), observed in closed PSII reaction centers (where photochemistry is absent) can be expressed as:

\[ \Phi'_o = \frac{k_r}{k_r + k_b} \]  

(14)

Eqns. (13) and (14) allow expressing \( \Phi'^{ext} \) as a function of fluorescence yields:

\[ \Phi'^{ext} = \frac{\Phi'_r - \Phi'_o}{\Phi'_o} = \frac{F_r - F_o}{F_r} = \frac{F_r - F_s}{F_m} \]  

(15)

where \( F_s \) is the variable fluorescence. Combining Eqns. (1) and (15) yields

\[ \Phi'^{ext} = \frac{F_s}{F_m} - \frac{a_{PSII}(\lambda)}{a_{PSII}(\lambda)} \]  

(16)

allowing calculation of the optical absorption cross section of the PSII unit from measurements of variable fluorescence and the functional absorption cross section.

In general, FRR excitation protocol can last from several tens of microseconds to several tens of seconds, and can be performed with different levels of excitation energy. Here we define two types of excitation protocols implemented in the submersible FRR instrument: saturation protocol, performed over 100-200 \( \mu \)s, with high excitation energies, and relaxation protocol, performed over 1-10 ms with low excitation energies. Saturation protocol is used to cumulatively reduce \( Q_{a} \) within 40-80 \( \mu \)s, during which time the probability of \( Q_{a} \) reoxidation and subsequent reduction by a second photochemical turnover is negligible. To achieve this goal, a series of 50 to 100 flashes is applied, each 0.25 - 1 \( \mu \)s in duration at 2 \( \mu \)s intervals, and pulse power of up to 0.01 moles quanta m\(^{-2}\) s\(^{-1}\). The fluorescence signal observed during saturation protocol increases from \( F_o \) (or \( F_r \), when measured under ambient irradiance) to \( F_m \) with a rate proportional to functional absorption cross section. The saturation protocol is used to measure \( F_o \)
and $F_n$ ($F'$ and $F''$ when measured under ambient irradiance), and to assess the functional absorption cross section of PSII.

Relaxation protocol is used to assess the rates of electron transport within PSII. It is applied immediately following the saturation protocol, as a series of 40 to 80 flashes at time intervals controlled from 50 μs to 200 μs. During the relaxation protocol the rate of excitation delivery to RCII is far slower that the rate of electron transport from $Q_A$ to $Q_B$. As a result, the fluorescence yields decrease with a kinetics corresponding to that of $Q_A$ reoxidation. Although controlled by two different phenomena ($Q_A$ reduction by the excitation energy, and reoxidation by a forward electron transport), fluorescence changes during the saturation + relaxation protocol is described by a common expression (Eqn. 1 to 7), and all the photosynthetic parameters are retrieved by fitting the whole fluorescence transient into a common set of parameters. In a following section we discuss the experimental fluorescence transient acquired during saturation and relaxation protocols.

1.3. Experimental protocols in Fast Repetition Rate Fluorometry

The rate of fluorescence increase observed during saturation protocol is controlled by the excitation energy and functional absorption cross section. To investigate the relationship between excitation energy and the fluorescence response, a series of 60 flashes of 0.25 μs to 1.0 μs duration, at 2 μs intervals was applied (Fig. 2). At increasing excitation energies the fluorescence yield saturates at a faster rate, reaching the $F_n$ level within 60 μs (Fig. 2A). When plotted as a function of the cumulative excitation energy (Fig. 2B), however, all the fluorescence profiles display the same saturation character within the first 120 μs of the excitation protocol. The fluorescence saturation profile deviates from the first order kinetics, suggesting energy transfer between PSII units. The consistent character of these profiles, irrespective of the energy of the excitation flashes, suggests that the rates of PSII photochemistry significantly exceeded the rates of $Q_A$ reoxidation. Assuming that g(θ) = 1 (Eqn 6), the calculated $F_n$, $F_m$, and $σ_{sat}$ are independent of excitation energies (Table 1).
Figure 2. Effect of excitation energy on fluorescence saturation profiles observed during saturation protocol. The average excitation energy was controlled by varying the flash duration from 0.25 µs to 1.0 µs, resulting in increasing rates of fluorescence saturation (A). When plotted as a function of cumulative excitation energy (B), however, all the saturation profiles displayed similar character, suggesting that the effects of Q* reoxidation on the fluorescence saturation profile are negligible within the range of excitation energies used.

Table 1. Calculation of \( F_0 \), \( F_\infty \), and \( \sigma_{psu} \) from saturation protocols with different excitation energies (Fig. 2A, B).

<table>
<thead>
<tr>
<th>Flashlet length [µs]</th>
<th>Excitation energy [quanta/RCII]</th>
<th>( F_0 )</th>
<th>( F_\infty )</th>
<th>( \sigma_{psu} ) [Å²]</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4.11</td>
<td>2427</td>
<td>6652</td>
<td>281</td>
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<tr>
<td>0.75</td>
<td>2.97</td>
<td>2423</td>
<td>6630</td>
<td>293</td>
</tr>
<tr>
<td>0.5</td>
<td>1.93</td>
<td>2435</td>
<td>6626</td>
<td>294</td>
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<tr>
<td>0.25</td>
<td>1.18</td>
<td>2447</td>
<td>6606</td>
<td>302</td>
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reoxidation, due to reduction of the PQ pool. This example clearly demonstrates the dependence of the $Q_{N}$ reoxidation rates on the level of photosynthetic activity within PSII, particularly the level of PQ pool reduction.

Figure 3. FRR fluorescence transients observed during saturation-relaxation protocol. (A) Saturation protocol is performed with flashlet duration of 0.8 µs, and time interval of 1.0 µs, while relaxation protocol is performed with 40 flashlet at 20 µs intervals, with the excitation energies varied from 0.002 to 0.022 quanta/RCl. Continuous lines represent theoretical fit with parameters shown in Table 2. Dotted lines represent cumulative excitation energies in units of quanta/RCl. (B) Extended relaxation protocol, where the initial 40 flashlets at 20 µs intervals are followed by 500 flashlets at 200 µs intervals.
The flash energies selected for saturation protocol represent a compromise between the requirement of reducing $Q_a$ within the single electron turnover time, while minimizing the possible effect of the fluorescence quenching in PSII reaction centers by formation of a carotenoid triplet (Breton, Gesmundo et al. 1979), or $P_{700}$ accumulation (Butler 1972), (Sontjeveld, Rademaker et al. 1979), (Deprez, Dobek et al. 1983). No significant changes in $F_{m}$ nor in the shape of the fluorescence saturation profile was observed at varying excitation energies, suggesting that these quenching phenomena do not contribute significantly to the fluorescence signal observed during saturation protocol.

1.4. FRR relaxation protocol

Following a single saturation flash, the fluorescence yield decreases with three-four exponential kinetics, with the fastest component in the range of 150-300 µs, a medium component in the range of 600-2000 µs, and the remaining one or two slow component in the range of 30 ms to several seconds. This kinetics is measured during relaxation protocol, designed as a series of flashlets applied at long time intervals. Because of the finite excitation energy, the relaxation protocol will invariably reexcite a portion of PSII reaction centers, elevating the observed fluorescence decay transient above the "intrinsic" level that would be observed if $Q_a$ reoxidation were undisturbed by relaxation flashlets. Also, the reexcited reaction centers are likely to display kinetics of $Q_a$ reoxidation that is different from single-electron transfer.

To account for the self-excitation effects, the entire fluorescence transient (saturation +relaxation protocol) is fitted into a common model (Eqn. 7). Nevertheless, as the length of the relaxation protocol increases beyond 5 to 10 ms, and the number of electrons passed from $Q_a$ onto secondary electron acceptors increases, the measured kinetics of the $Q_a$ reoxidation becomes protocol dependent. Therefore, interpretation of the kinetics of $Q_a$ reoxidation using FRR method requires clear specification of the excitation protocol (e.g. excitation energy and the protocol length) employed in the relaxation sequence.

When the early phase of $Q_a$ reoxidation is of interest, (i.e. when assessing $F_{m}$, $F_{i}$, and $q_{ps}$), it is sufficient to follow the fluorescence relaxation during the first 1-4 ms following the saturation flash. In Fig. 3A we show fluorescence profiles with relaxation sequence selected as a series of 40 flashlets at 20 µs time interval, with energies varying from 0.002 to 0.02 quanta/RCIIflashlet (0.08 to 0.8 quanta/RCII total). To observe further phases of fluorescence reoxidation, this protocol may be extended by a series of 500 flashlets at 200 µs delay (Fig. 3B), lasting for about 100 ms.

With increasing excitation energy, the fluorescence yield in relaxation protocol declines at slower rate. This phenomenon can be ascribed to both the self-excitation by the relaxation flashlets, and to increasing probability of reoxidation of $Q_a$ with a second electron. At flashlet energies above 0.02 quanta/RCII, the extended relaxation profile displays a secondary rise following absorption of about 15-20 quanta per RCII, indicating a decrease in the rates of $Q_a$. 
To quantify the effect of the excitation energy on the measured kinetics of $Q_a^-$ reoxidation, the fluorescence transients measured with varying excitation energies were fitted into Eqs. 7 and 8, within 1 ms and 40 ms time window following saturation protocol. Results presented in Table 2 show almost identical set of the calculated $F_n$, $F_m$, and $\alpha_{out}$ values, irrespective of the excitation energies in the relaxation protocol and the selected time window. Calculated kinetics of $Q_a^-$ reoxidation, when analyzed over 1 ms window, can be resolved into two components (Table 2), with almost identical parameters independent of the excitation energy. When analyzed within 40 ms time window, this kinetics can be resolved into three components, where the shortest time constant increased by about 11%, and the longest time constant decreased by about 22% within the 0.002 to 0.01 q/RCII/flashlet range of excitation energies. Different kinetics of $Q_a^-$ reoxidation calculated over 40 ms time window at varying excitation energies most likely reflect changes in the population of reduced electron carriers ($Q_a^-, Q_b^-$, PQ pool) as increasing numbers of electrons are passed from QA' to the electron transport chain.

Table 2. Fluorescence parameters and time constants of $Q_a^-$ reoxidation measured at different excitation energies applied in the relaxation protocol (Fig. 3A, B), analyzed with double-exponential model of $Q_a^-$ reoxidation kinetics within 1 ms time window, and with triple-exponential model of $Q_a^-$ reoxidation analyzed within 40 ms time window

<table>
<thead>
<tr>
<th>Energy [q/RCII/flashlet]</th>
<th>$F_n$</th>
<th>$F_m$</th>
<th>$\alpha_{out}$</th>
<th>$\tau_1$ [ns]</th>
<th>$\alpha_1$</th>
<th>$\tau_2$ [ns]</th>
<th>$\alpha_2$</th>
<th>$\tau_3$ [ns]</th>
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<td>Two-exponential calculations of $Q_a^-$ reoxidation over 1 ms time window</td>
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<td></td>
<td></td>
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<tr>
<td>0.002 2035 6097 308</td>
<td>0.389</td>
<td>264</td>
<td>0.602</td>
<td>1423</td>
<td>-----</td>
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<tr>
<td>0.0036 2040 6048 327</td>
<td>0.384</td>
<td>269</td>
<td>0.616</td>
<td>1543</td>
<td>-----</td>
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<tr>
<td>0.0064 2055 6038 332</td>
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<td>0.623</td>
<td>1499</td>
<td>-----</td>
<td>-----</td>
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<tr>
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<td>0.378</td>
<td>289</td>
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<td>1511</td>
<td>-----</td>
<td>-----</td>
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<tr>
<td>0.0221 2008 5973 310</td>
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<td>1582</td>
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<td>Three-exponential calculations of $Q_a^-$ reoxidation over 40 ms time window</td>
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<td>0.0126 2009 6000 309</td>
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<td>0.0221 2005 5998 311</td>
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<td>0.479</td>
<td>1276</td>
<td>0.230</td>
<td>12.4</td>
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To make the measurements of $Q\text{,}$ reoxidation as protocol independent as possible, the excitation energy and the time window in relaxation protocol should be selected to allow a single electron transport. In situation where the kinetics of electron transport from $Q\text{,}$ to $Q\text{,}$ is of interest, it is sufficient to perform the relaxation protocol within 1 to 4 milliseconds following saturation protocol, which usually satisfies this requirement.

2. Assessment of photosynthetic rates using FRR data.

Photosynthetic parameters measured using FRR fluorometry, such as chlorophyll variable fluorescence, functional and optical absorption cross sections, rates of electron transport within PSII, and photochemical/nonphotochemical quenching, together with PAR, can be used to estimate primary production. Some of these parameters, particularly $F/F$ in conjunction with PAR, have been used extensively to calculate the photosynthetic rates (Genty, Briantais et al 1989). In more extensive approaches, functional absorption cross section, together with photochemical quenching can be combined into more robust models relating photochemistry and fluorescence. (Kolber and Falkowski 1993). Technical capability of measuring a whole suite of photosynthetic parameters allowed by FRR fluorometry is likely to further expand the methodology for in situ assessment of primary production. Therefore, our goal here is not to recommend or endorse any particular methodology or approach, but rather to demonstrate the relevance of the photosynthetic parameters measured by FRR fluorometry for estimating of primary production.

Knowing $\sigma_{PSII}$ it is possible to calculate the rate of electrons transport in PSII reaction centers:

$$J_{PSII} = E \sigma_{PSII} Q = E \sigma_{PSII} \frac{F_m - F}{F_m - F_o}.$$  \hspace{1cm} (17)$$

or

$$J_{PSII} = E \sigma_{PSII} \frac{F_m - F}{F_m} = E \sigma_{PSII} \frac{F_m - F}{F_o}.$$  \hspace{1cm} (18)$$

where $E$ is the ambient irradiance, and $Q$ is the photochemical quenching induced by the ambient irradiance. To estimate the bulk rates of electron transport, or gross photosynthetic rates, Eqs. (17) or (18) have to be multiplied by a concentration of the functional reaction centers, $n_{PSII}$.
\[ J^* = E \alpha_{\text{PSII}} \frac{F_m - F'}{F_m - F} \eta_{\text{PSII}} \]  

(20)

Estimation of \( \eta_{\text{PSII}} \) presents a relatively complex problem (Ko~l~ber and Falkowski 1993), nevertheless, as only the functional PSII reaction centers do contribute to variable fluorescence, a proportional relationship between \( F_m \) and \( \eta_{\text{PSII}} \) may be postulated, leading to expression:

\[ J^* = E \alpha_{\text{PSII}} \frac{F_m - F'}{F_m - F} k F_r = E \alpha_{\text{PSII}} \frac{F_m - F'}{k}, \]  

(21)

where \( k \) is a proportionality factor between \( F_r \) and \( \eta_{\text{PSII}} \). In the above notation, the product \( (F_m-F')k \) can be interpreted as a measure of the concentration of functional PSII reaction centers that are open under ambient irradiance \( E \). Further improvements in the presented algorithm will require quantitation of the \( k \) constant in Eqn. (21), evaluating the effects of nutrients/trace metal limitation on photosynthetic performance of phytoplankton, assessing the effects of non-photochemical quenching on the photosynthetic rates, and quantifying the effects of photoinhibition at supersaturating irradiances.

Equations (17) to (21) are valid under subsaturating irradiances. At supersaturating irradiance photosynthetic rates are limited by the turnover time of photosynthesis (Ko~l~ber and Falkowski 1993).

The fluorescence-based estimates of photosynthetic rates represent a gross primary production. Calculation of oxygen evolution, or carbon fixation-equivalent primary production will require assessing of the respiration rates and the photosynthetic quotient.
3. Example of field data acquired using Fast Repetition Rate fluorometry

SJ9608, Stat 52

\[
\text{PAR (µE m}^2\text{s}^{-1}), \sigma_{\text{PAR}}
\]

\[
\begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 & 600 \\
\end{array}
\]

\[
\begin{array}{c}
F_0, F_m, F_m \text{ (dark)} \\
\end{array}
\]

\[
\begin{array}{c}
\sigma_{\text{PAR}} \\
\sigma_{\text{PAR}} = (F_m F)_0 (F_m F_0) \\
\end{array}
\]

\[
\begin{array}{c}
0 & 500 & 1000 & 1500 & 2000 & 2500 & 3000 & 3500 & 4000 \\
\end{array}
\]

\[
\begin{array}{c}
\text{Depth (m)} \\
\end{array}
\]

\[
\begin{array}{c}
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 \\
\end{array}
\]

\[
\begin{array}{c}
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 \\
\end{array}
\]

SJ9608, Stat 50

\[
\text{PAR (µE m}^2\text{s}^{-1}), \sigma_{\text{PAR}}
\]

\[
\begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 & 600 \\
\end{array}
\]

\[
\begin{array}{c}
F_0, F_m, F_m \text{ (dark)} \\
\end{array}
\]

\[
\begin{array}{c}
\sigma_{\text{PAR}} \\
\sigma_{\text{PAR}} = (F_m F)_0 (F_m F_0) \\
\end{array}
\]

\[
\begin{array}{c}
0 & 500 & 1000 & 1500 & 2000 & 2500 & 3000 & 3500 & 4000 \\
\end{array}
\]

\[
\begin{array}{c}
\text{Depth (m)} \\
\end{array}
\]

\[
\begin{array}{c}
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 \\
\end{array}
\]

Figure 4. Example of FRR data acquired in July 96 in coastal North Atlantic. STAT52 profile was acquired during the night, while STAT50 profile was acquired in the middle of the day. The open symbols represent the fluorescence signals measured in the light-exposed sample chamber, closed symbols represent the fluorescence signals measured in the dark sample chamber. When measured during the day, the photochemical quenching decreases to 0.2 at the surface, indicating closing of PSII reaction centers by ambient irradiance. Chlorophyll maximum at 25 m was about 3.6 µg/l in STAT52 profile and 3.1 µg/l in STAT50 profile.
Example of the FRR data acquired in July 96, in coastal North Atlantic are presented in Fig. 4. The FRR fluorescence profile recorded during the day (STAT52) displays a decrease in the variable fluorescence at irradiances where rates of excitation delivery to PSII reaction center, calculated as a product of $E$ and $\sigma_{\text{max}}$, becomes comparable with the turnover time of photosynthesis. As a result, the fraction of closed PSII reaction centers increases, and the fluorescence yield observed under background irradiance, $F'$, increases above the $F_o$ level:

$$F' = F_o + (F_m - F_o) C(E),$$

(22)

where $C(E)$ is a fraction of reaction centers closed by ambient irradiance:

$$C(E) = \frac{E \sigma_{\text{PSII}}}{E \sigma_{\text{PSII}} + \frac{1}{r_{\text{QA}}}},$$

and $r_{\text{QA}}$ is the rate of QA reoxidation. In darkness $r_{\text{QA}}$ averages at 0.6 to 1 ms, but increases to about 5 to 10 ms when PQ pool becomes reduced. Ultimately, the $F'$ signal approaches $F_o$ at supersaturating irradiances, and the photochemical quenching decreases to 0.15 - 0.20. The lowest limit of photochemical quenching measured using a single saturating flash is determined by the level of electron cycle around PSII (Falkowski, Fujita et al. 1986).

Sections of photosynthetic parameters, together with temperature, salinity, and nutrients distribution, measured along the transect from 35.30 N 75.42 W to 35.30 N 74.68 W in July 1996 are shown in Fig. 5. Note a high level of spatial correlation between Temperature, Salinity, and Chlorophyll distribution. Variable fluorescence measured with FRR fluorometer corresponds well to distribution of extracted chlorophyll $F_o/F_m$ signal, as well as functional absorption cross section are spatially correlated with nutrients distribution, indicating a close coupling between nutrients and photosynthetic viability of phytoplankton.
Figure 5. Sections of Temperature, Salinity, nutrients distribution, and photosynthetic parameters, measured along the transect from 3S to 3N 42 W to 35 40 N 74 68 W in July 1996.
FRR fluorometry was used in two IRONEX experiments. Although the iron enrichment performed during IRONEX I did not produce significant increase in chlorophyll biomass and carbon fixation, FRR measurements clearly demonstrated a transient increase in quantum yield of photosynthesis by a factor of two (Kolber, Barber et al. 1994) (Fig. 6). The increase in the quantum yield of photosynthesis in the iron patch coincided with increase in chlorophyll biomass estimated by acetone chlorophyll extraction from water samples collected at discrete depths.

![Graph showing quantum yield of photosynthesis](image1)

**Figure 6.** Response in quantum yield of photosynthesis ($F_v/F_m$) following iron addition during IRONEX I experiment. Both the chlorophyll biomass and quantum yield of photosynthesis increased by a factor of two inside the iron patch. Due to low chlorophyll concentration, the profiles of $F_v/F_m$ in the water column were smoothed using a 5 m long running average.

![Graph showing phytoplankton response to iron enrichment](image2)

**Figure 7.** Transient character of phytoplankton response to iron enrichment during IRONEX I experiment. Photosynthetic parameters shown here represent an average over the first 25 m of the water column.
indicating that F/F_m can be used as an indicator of phytoplankton growth rates.

Despite of a significant increase in photosynthetic yields, the chlorophyll biomass increased by no more than a factor of three over the length of the whole experiment. The reason for this apparent “failure” of IRONEX I is shown in Fig. 7, where FRR data revealed a transient character of phytoplankton response, peaking on the second day following iron addition, but then decreasing sharply to the initial conditions. It was concluded that iron was removed from the euphotic zone too quickly to be assimilated by phytoplankton.

Based on these observations, iron addition in IRONEX II was performed in three separate allotments at 3 days time intervals. Despite of similar amount of iron used, a delayed release resulted in much longer persistence of iron in the water column, elevated photosynthetic yields [Fig. 8] and about 20 times increase in the chlorophyll biomass (Behrenfeld, Bale et al. 1996).
References


