Fluorescence quenching analysis

Author:
Jun Minagawa

Affiliation:
Institute of Low Temperature Science, Hokkaido University, N19 W8, Sapporo 060-0819, Japan

Name and address for correspondence:
Jun Minagawa
Institute of Low Temperature Science
Hokkaido University
N19 W8, Sapporo 060-0819
Japan
TEL: +81-(0)11-706-5471
FAX: +81-(0)11-706-5471
E-mail: minagawa@lowtem.hokudai.ac.jp
A. Introduction

Chlorophyll a fluorescence is a highly versatile tool, not only for researchers studying photosynthesis, but also for those working in broader fields related to physiology of plants and green algae. Chlorophyll fluorescence analysis is sensitive, real-time, non-invasive, and relatively simple, but indirect. The section here is meant to guide proper interpretation and familiarize the reader with terminology (for further reading see Maxwell and Johnson, 2000; Falkowski and Raven, 2007). Chlorophyll fluorescence analysis is applicable to chapters 15, 16 and 22 in *The Chlamydomonas Sourcebook, Volume 2*.

Absorption of a photon raises a chlorophyll a molecule to its lowest singlet excited state, for which three internal decay pathways exist: fluorescence, in which the molecule returns to the ground state with the emission of radiation; internal conversion, in which the energy of the molecule is converted into vibrational energy; and intersystem crossing, in which the singlet state is converted to the triplet state. If certain other molecules are present along with the chlorophyll, external decay pathway(s) may also become available in addition to the internal decay pathways. Such external pathways facilitate the transfer of energy to a molecule with a similar energy gap or the transfer of an electron to or from another molecule, such as in excitation energy transfer in light-harvesting antennae and charge separation in photochemical reaction centers, respectively. All of these downward processes competitively contribute to the decay of the chlorophyll excited state. Accordingly, an increase in the rate of one of these processes would increase its share of the decay process and lower the fluorescence yield ($\phi_f$).

The quantum yield of chlorophyll fluorescence from the photosynthetic apparatus is therefore 0.6-3%, while chlorophyll a in an organic solvent exhibits a high fluorescence yield of approximately 30% (Latimer et al., 1956; Trissl et al., 1993). Historically, the term “quenching” refers to all processes that lower $\phi_f$. 
B. Quenching analysis

Classic experiments showing the relationship between photosynthetic reactions and Φ_r were made by Kautsky and Hirsh (1931). They recorded a rapid rise of fluorescence from a leaf through colored glass at the onset of illumination, which was followed by a decline to reach a steady level (Kautsky effect). Duysens and Sweers (1963) proposed that changes in Φ_r are primarily related to the redox state of the electron-transferring components of PS II, and introduced the term Q as a quencher. Butler and Kitajima (1975) elaborated that with Q in the ground state, charge separation can occur and the reaction center is described as being open. When Q is reduced, further stable charge separation is inhibited and the center is described as being closed. Subsequent experiments have shown that Q is identical to QA in the PS II reaction center.

Photosynthetic reactions occur in PS II over a broad time range. Reactions lasting up to hundreds of picoseconds are considered to be “ultrafast” reactions; these include excitation energy transfer and charge separation and stabilization, which have been described by the “exciton-radical pair equilibrium” model or its derivatives (Schatz et al., 1988; Laible et al., 1994; van Grondelle et al., 1994). The PS II acceptor-side reactions that include electron sharing between the primary and secondary quinones of PS II (QA and QB respectively), the subsequent reactions that result in the reduction of the intersystem electron carriers, and the PS II donor-side reactions that include electron donation from the oxygen-evolving complex to the secondary electron donor in PS II (Yz) and from Yz to P680+, are considered to be the “fast” reactions; these last from tens of microseconds to tens of milliseconds. Lastly, energy-dependent qE-quenching, state transitions, and photoinhibition are considered to be the “slow” more acclimatory reactions; the duration of these reactions ranges from a couple of seconds to hundreds of minutes. Different and specialized techniques have been applied for elucidating the
reactions in different time domains. Only a brief summary of fluorescence quenching analysis is provided here, which targets the “slow” reactions.

C. Photochemical quenching

If all antenna chlorophylls for PS II are similar in their energy levels, the probability of excitation decay is independent of the individual pigment. In a simple scheme, the quantum yield of fluorescence ($\phi_f$) and photochemistry ($\phi_{II}$) are therefore given by the rate constants for fluorescence ($k_f$) and photochemistry ($k_p$), respectively, divided by the sum of the rate constants for all competing processes of de-excitation:

$$\phi_f = \frac{k_f}{k_f + k_p + k_h}$$

$$\phi_{II} = \frac{k_p}{k_f + k_p + k_h}$$

where $k_h$ denotes the rate constant for the thermal dissipation. When $Q_A$ is in its oxidized state, the charge stabilization reaction ($P680^+\text{Pheo}Q_A \to P680^+\text{Pheo}^-Q_A \to P680^+\text{Pheo}Q_A^-$) occurs within hundreds of picoseconds (Renger et al., 1995), which primarily results in the loss of the “exciton-radical pair equilibrium” state. Thus, the pathway of photochemistry in the open PS II reaction center results in a decrease of $\phi_f$, which is termed photochemical quenching. When $Q_A$ is in its reduced state, i.e. when the centers are closed, the charge separated state ($P680^+\text{Pheo}$) cannot be stabilized and the value of $k_p$ is reduced to zero. The “exciton-radical pair equilibrium” state then continues where the loss of the excited state would primarily be due to fluorescence.

The two levels of fluorescence are defined as follows: Fo (unquenched minimal yield of fluorescence) is the level of chlorophyll fluorescence when all PS II centers are open and Fm (unquenched maximal yield of fluorescence) is the maximal level of chlorophyll fluorescence when all PS II centers are closed. Practically, Fo and Fm are measured as fluorescence emitted from dark-adapted samples when probed with a weak pulse of measuring light before and after
a strong saturating flash is given, respectively. The difference between Fm and Fo is defined as Fv (unquenched variable fluorescence).

D. Non-photochemical quenching

Overexcitation of chlorophyll and overreduction of the electron transport chain can result in increased generation of reactive intermediates and harmful byproducts of photosynthesis. In order to maintain a short lifetime of an excited chlorophyll molecule and protect the organism from damage by excess light absorption, there exist additional decay pathways for de-excitation of chlorophyll molecules in competition with photochemistry, fluorescence, and other decay processes. Fluorescence quenching processes observed under such conditions are collectively called non-photochemical quenching and have been described for a long time (Govindjee et al., 1967; Bonaventura and Myers, 1969; Murata, 1969; Murata and Sugahara, 1969). Classically, mechanisms for inducing non-photochemical quenching have been categorized into 3 classes; 1) high-energy dependent “qE-quenching”, 2) state transition-dependent “qT-quenching”, and 3) photoinhibition-dependent “qI-quenching” (Krause and Weis, 1991). All these processes have been postulated to have physiological roles that contribute to the protection of the photosynthetic apparatus. By comparing fluorescence quenching in samples with or without the suppression of photochemistry, it is possible to distinguish whether it is based on a change in k_p or k_h, assuming that the intrinsic rate constant for fluorescence (k_f) is not variable.

E. Pulsed-amplitude modulation (PAM) fluorometry

In order to distinguish between the photochemical and non-photochemical contributions to quenching, the so-called “light doubling” or “saturation pulse” method was introduced (Bradbury and Baker, 1981). This technique is based on the assumption that all PS II reaction centers can be transiently closed, thus eliminating the contribution of all photochemical quenching without causing any change in non-photochemical quenching. An innovative device in the application of chlorophyll fluorescence uses a “modulated” measuring beam (Quick and
Horton, 1984), where the fluorescence signals are isolated by a detector with a lock-in amplifier, making the accurate separation of signals in the presence of a background light possible. Based on these ideas, “pulsed amplitude modulation (PAM)” fluorometry was designed (Schreiber et al., 1986). Using this system, fluorescence yield can be measured even in the presence of white background illumination and, most significantly, in the presence of sunlight. In order to close all PS II reaction centers transiently, a high-intensity, short-duration flash of light that lasts for approximately 0.2–1 seconds is given. During this saturation pulse, the fluorescence yield reaches a value equivalent to that which would be obtained in the absence of any photochemical quenching. Of particular importance is that Fm’ (quenched maximal yield of fluorescence) and Fo’ (quenched minimal yield of fluorescence) can be readily measured in addition to that of the dark-adapted state, Fm and Fo.

**F. Video imaging analysis**

The versatility of chlorophyll fluorometry increased significantly with the development of techniques that take advantage of CCD (Charge-Coupled Device) cameras and computerized data processing (Omasa et al., 1987; Fenton and Crofts, 1990; Nedbal et al., 2000; Oxborough, 2004). The typical instrument is illustrated in Fig. 1, with which spatial and temporal changes of various fluorescence parameters can be readily obtained. This has facilitated screens for photosynthetic mutants of various organisms including *Chlamydomonas* (Bennoun and Béal, 1997; Niyogi et al., 1997; Fleischmann et al., 1999; Kruse et al., 1999), and allowed comparisons of photosynthetic performance on different leaf spots (Daley et al., 1989; Siebke and Weis, 1995).
CCD camera

red filter

halogen lamp (actinic light)

blue filter

Xenon lamp (saturating flashes)

blue filter

orange LEDs (measuring flashes)

computer
G. Parameters used in quenching analysis

Calculation of fluorescence parameters is best explained by referring to a typical experimental trace obtained with a PAM-type fluorometer (Fig. 2).

Measurement is initiated by switching on the weak measuring light, providing a measure of Fo (upward arrow). A saturating flash of light or multiple subsaturating flashlets that together trigger single turnover excitation (filled arrowheads), is then applied and allows the measurement of Fm from the dark-adapted sample. Next, actinic light (open bar) is applied and further saturating pulses are given at appropriate intervals to measure Fm'. The transient fluorescence, Ft, is monitored during the entire duration. The fluorescence minimum in the quenched state, Fo', is measured immediately after the removal of actinic light (downward arrow). The variable fluorescence in the dark and in the quenched state (Fv') is expressed by Fv.
= Fm – Fo, and Fv’ = Fm’ – Fo’, respectively. Resolution of quenching parameters provides a variety of information on the functional state of the photosynthetic apparatus as described below. These parameters are the ones used most often in the literature.

1. Fo’

Fo’ is the minimal yield of fluorescence from samples in the “quenched” state and is usually measured immediately after the removal of actinic light, so that the fluorescence reflects the nature of the samples in the actinic light at a time when all QA is oxidized. By comparing Fo’ with Fo, one can estimate if the fluorescence quenching observed in Fm’ is due to quenching in a portion of Fv or Fo. Measurement of this parameter is sometimes problematic, as a large proportion of non-photochemical quenching quickly follows changes in the ΔpH, which also decays rapidly once the illumination is removed. To quantify this potential source of artifacts, Oxborough and Baker (1997) estimated Fo’ through a simple equation involving only Fo, Fm, and Fm’. The formalization is as follows:

\[
\text{minimal fluorescence (quenched): } Fo' = \frac{Fo}{Fv} \frac{Fo}{Fm + Fm'}
\]

They demonstrated that the calculated value of Fo’ correlates well with the experimentally measured Fo’ (Oxborough and Baker, 1997).

2. Quantum yield of photochemistry (Fv / Fm, Fv’ / Fm’, ΔF / Fm’, φII)

Probably the most useful fluorescence parameter measures the proportion of absorbed energy used in photochemistry, namely the quantum yield of photochemistry. If measured in the dark, the intrinsic quantum yield is expressed as Fv / Fm (Kitajima and Butler, 1975). In the presence of light, the intrinsic and effective quantum yields are calculated as Fv’ / Fm’ and ΔF / Fm’, respectively (Genty et al., 1989). φII has been used for quantum yield of photochemistry in general, but, in a more limited sense, it is used for effective quantum yield in the light.
intrinsic quantum yield after dark adaptation: \[ \frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \]

intrinsic quantum yield during illumination: \[ \frac{F_v'}{F_m'} = \frac{F_m' - F_o'}{F_m'} \]

effective quantum yield during illumination: \[ \phi_{II} = \frac{\Delta F}{F_m'} = \frac{F_m' - F_t}{F_m'} \]

The effective quantum yield in the light (\(\phi_{II}\)) is correlated with the quantum yield of CO\(_2\) assimilation under laboratory conditions, where linear electron flow is dominant (Fryer et al., 1998). This parameter is the easiest one to measure; a measurement can be performed by simply applying a fluorometer to a sample and measuring fluorescence before and after a saturating pulse (\(F_t, F_m'\)). The dark-adapted values of \(F_v / F_m\) are sensitive indicators of a maximal photosynthetic performance, with optimal values of around 0.832 measured for most plant species (Björkman and Demmig, 1987). Sustained heat dissipation with a low \(F_v / F_m\) is often seen when a sample has been exposed to photoinhibitory conditions. If photochemical efficiency of a plant was temporarily suppressed due to reversible non-photochemical quenching, the intrinsic quantum yield would be decreased only in the light, e.g. \(F_v' / F_m'\) would be lowered while \(F_v / F_m\) would remain the same.

3. Proportion of open PS II centers (\(q_P, q_L\))

Another widely used fluorescence parameter is photochemical quenching, \(q_P\). This is calculated as:

\[
\text{proportion of open PS II centers} : q_P = \frac{F_m' - F_t}{F_m' - F_o'} = \frac{\Delta F}{F_v'}
\]

While \(\phi_{II}\) is the proportion of absorbed energy used in photochemistry, \(q_P\) indicates the proportion of open PS II centers. The alternative expression, 1- \(q_P\), reflects the proportion of closed centers, which is sometimes termed the “excitation pressure” on PS II (Maxwell et al., 1994).

\[
\text{proportion of closed PS II centers (excitation pressure)} : 1 - q_P
\]
q_P and φ_{II} can be interrelated by Fv' / Fm' (Oxborough and Baker, 1997):

\[
\phi_{II} = \frac{\Delta F}{Fm'}
= \frac{\Delta F \cdot Fv'}{Fv' \cdot Fm'}
= q_P \cdot \frac{Fv'}{Fm'}
\]

It is of note that while \( \phi_{II} \) is related to the overall efficiency of photochemistry, \( q_P \) and \( Fv' / Fm' \) provide information about the underlying processes. A decrease in \( q_P \) is due to an increase in the proportion of closed PS II centers, which could be brought about by any defects in the components downstream of PS II, such as inhibition in PS I. A decrease in \( Fv' / Fm' \), on the other hand, can be caused by any defect in the quantum yield of PS II itself, such as non-photochemical quenching. Either case could decrease \( \phi_{II} \).

The parameter \( q_P \) is based on the so-called “puddle” antenna model, where each PS II center possesses its own independent antenna system. However, accumulating evidence has suggested that the real photosynthetic units are connected by shared antennae, the “lake” antenna. Therefore, Kramer et al. (2004) extended the approach by Joliot and Joliot (1964), who had described the non-linear relationship of concentration of Q_A and variable fluorescence, and derived a parameter \( q_L \) to represent a realistic estimate of the fraction of open PS II centers with a high connectivity of PS II units.

\[
q_L = \frac{Fm' - Ft}{Fm' - Fo'} \cdot \frac{Fo'}{Ft}
= q_P \cdot \frac{Fo'}{Ft}
\]

**4. Electron transfer rate (ETR)**

Since \( \phi_{II} \) is the quantum yield of PS II photochemistry, it can be used to calculate the rate of linear electron transfer (ETR; Genty et al., 1989):

\[
\text{electron transfer rate : } ETR = \phi_{II} \cdot PFDa \cdot 0.5
\]
where PFDa is absorbed light (µmol photon m$^{-2}$s$^{-1}$) and 0.5 is an assumed factor that accounts for the partitioning of energy between PS II and PS I. This assumption could be reasonable during steady state when the PS I and PS II turnover rates are equal. Care should be taken when one encounters conditions where cyclic electron transfer around PS I or PS II is predominant.

5. Non-photochemical quenching (NPQ, qN)

The study of energy dissipation processes has been complicated by the fact that researchers have used different terms for non-photochemical quenching, e.g. the parameters NPQ vs qN. The most straightforward way of quantifying non-photochemical quenching is by measuring the quenched fraction of maximal fluorescence, namely the Stern-Volmer-type equation (Bilger and Björkman, 1990):

\[
\text{non-photochemical quenching} : \text{NPQ} = \frac{F_m - F_m'}{F_m'}
\]

NPQ is linearly related to heat dissipation and lies on a scale from zero to infinity. In a typical photosynthetic organism, values might be expected in the range 0.5-3.5 at saturating light intensities; however, this varies markedly between species and acclimatization conditions.

When grown under normal light and temperature conditions, *Chlamydomonas* exhibits a low value of NPQ (Finazzi et al., 2006). Another term for quantifying non-photochemical quenching is qN (van Kooten and Snel, 1990):

\[
\text{non-photochemical quenching} : q_N = \frac{F_m - F_m'}{F_m - F_o}
\]

This parameter requires measurement of Fo and falls on a scale of 0-1 and is therefore relatively insensitive to changes in quenching at higher values.
References


