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REVIEW PAPER

# Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications

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## Abstract

**Chlorophyll fluorescence is a non-invasive measurement of photosystem II (PSII) activity and is a commonly used technique in plant physiology. The sensitivity of PSII activity to abiotic and biotic factors has made this a key technique not only for understanding the photosynthetic mechanisms but also as a broader indicator of how plants respond to environmental change. This, along with low cost and ease of collecting data, has resulted in the appearance of a large array of instrument types for measurement and calculated parameters which can be bewildering for the new user. Moreover, its accessibility can lead to misuse and misinterpretation when the underlying photosynthetic processes are not fully appreciated. This review is timely because it sits at a point of renewed interest in chlorophyll fluorescence where fast measurements of photosynthetic performance are now required for crop improvement purposes. Here we help the researcher make choices in terms of protocols using the equipment and expertise available, especially for field measurements. We start with a basic overview of the principles of fluorescence analysis and provide advice on best practice for taking pulse amplitude-modulated measurements. We also discuss a number of emerging techniques for contemporary crop and ecology research, where we see continual development and application of analytical techniques to meet the new challenges that have arisen in recent years. We end the review by briefly discussing the emerging area of monitoring fluorescence, chlorophyll fluorescence imaging, field phenotyping, and remote sensing of crops for yield and biomass enhancement.**

**Key words:** Chlorophyll fluorescence, field, imaging, phenotyping, photosynthesis, protocols, quantum efficiency.

## The aim of this review

Chlorophyll fluorescence is one of the most popular techniques in plant physiology because of the ease with which the user can gain detailed information on the state of photosystem II (PSII) at a relatively low cost. It has had a major role in understanding the fundamental mechanisms of photosynthesis, the responses of plants to environmental change, genetic variation, and ecological diversity. The aim of this review is to produce a guide to good practice in chlorophyll fluorescence analysis, and provide an introduction to and appreciation of some of the emerging technologies and new applications. Although we will provide a brief overview and explanation of the principles of chlorophyll fluorescence, we do not provide an in-depth review of the technique itself and the theory behind measurements, and we refer readers to several excellent reviews covering this (Krause and Weiss, 1991; Maxwell and Johnson 2000; Adams *et al.*, 2004; Logan et al 2007; Baker, 2008).

The recently increased interest in the use of chlorophyll fluorescence techniques has been mainly due to research in crop improvement and in particular for the screening of desirable plant traits and linking genomic information with phenological responses (Baker and Rosenqvist, 2004;

Furbank *et al.*, 2009). As such there is great demand for protocols for rapid screening of photosynthesis that give high-resolution information regarding a plant's status (often in relation to plant-based growth/development parameters and yield or stress; Montes *et al.*, 2007; Furbank *et al.*, 2009) and where a high number of measurements are required in a short time period. The choice of parameters that can be calculated from fluorescence data combined with the variety of instrumentation available can make it bewildering for the user. It is critical that the protocols used and parameters measured are appropriate for the particular individual study and the research questions asked, in order to ensure valid data are collected and the correct conclusion(s) drawn. The user is faced with an array of options including the type of measurement platform, instrument setting, the timing of a period of dark adaptation, and the conditions under which the plants are measured. Inexperience can lead to data that do not fully address the hypothesis being tested or that do not adequately cover the range of phenotypes that the plant is capable of expressing.

In this review, we recognize the requirements for the use of this technique in the fields of ecology and agriculture and especially where measurements must be rapid. In addition, the development of new equipment such as monitoring fluorometers which are intended for automated long-term assessment of photosynthesis and chlorophyll fluorescence imaging for phenotypic screening has offered new opportunities.

## What is chlorophyll fluorescence?

Chlorophyll within a leaf exists as pigment–protein complexes in PSII, PSI, and within the light-harvesting complexes (LHCs) associated with each of these reaction centres. Light energy absorbed by chlorophyll molecules can (i) drive photosynthesis (photochemistry); (ii) be re-emitted as heat; or (iii) be re-emitted as light (fluorescence) (Fig. 1). These three processes do not exist in isolation but rather in competition with each other. Thus the yield of chlorophyll fluorescence emission gives us valuable information about the quantum efficiency of photochemistry and heat dissipation. This is important for plant photosynthesis and ultimately productivity because photochemistry is used to provide energy and reducing power for $CO_2$ assimilation. At room temperature, we assume the variations in the fluorescence signal arise from PSII only and we ignore emission from PSI largely because the signal does not make a significant contribution below 700 nm (Butler, 1978; Pfündel, 1998; Baker, 2008)

As chlorophyll fluorescence is a measure of re-emitted light (in the red wavebands) from PSII, naturally this means that any ambient light can interfere with the measurement of fluorescence and thus many early systems had to be used in darkness and/or highly controlled light environments. This issue was overcome by the development of modulating systems where the light used to induce fluorescence (the measuring beam) is applied at a known frequency (modulated) and the detector is set to measure at the same frequency (Schreiber *et al.*, 1986). In this way the detector will only measure fluorescence that results from excitation by the measuring beam and will not permit interference from ambient light. The clear advantage of this is that measurements can be made without darkening the leaf. Non-modulated fluorescence has recently been re-gaining interest, and there are many devices available that offer the measurement of a number of parameters taken in the dark, for example by analysing fluorescence induction transients that occur during the application of a 1 s pulse from a state of darkness (Strasser *et al.*, 2004). There is current interest in the use of small hand-held devices based on continuous fluorescence for screening, where key measurements can be made with great accuracy and speed. There is a plethora of parameters that are based on physical events within PSII which claim to give detailed structural and functional information about PSII activity, antenna size, and electron transport (Strasser *et al.*, 2004). Such techniques are sensitive and are gaining support for use in crop phenotyping using simple parameters (Pask *et al.*, 2012). However, the continued lack of empirical support for the more complex parameters restricts meaningful interpretation, and we urge caution. This contrasts with the large body of data that provides a sound physiological framework for the modulated technique. In this review, we only have the capacity to cover modulated fluorescence analysis.

## Principles of chlorophyll fluorescence analysis

This section describes the biochemical events that occur within the thylakoid membrane that are relevant for understanding chlorophyll fluorescence analysis. It is intended to help the reader understand the principles, while a detailed explanation of the parameters is given in the next section.

The state of reduction and oxidation (redox) state of key electron carriers is important when understanding the events that lead to changes in chlorophyll fluorescence, and this is described in Fig. 1. When light sufficient to drive photosynthesis is applied to a leaf after a period of darkness, there is a transient rise (usually for a few seconds) in the level of chlorophyll fluorescence that is usually the result of the reduction of electron carriers in the thylakoid membrane. The special chlorophyll in PSII, $P_{680}$, ejects an electron derived from water splitting to the electron acceptor $Q_A$ (a bound quinone) via the initial acceptor pheophytin. However, $Q_A$ is not able to accept another electron from $P_{680}$ until it has passed its electron to the next carrier, $Q_B$. In this state, the reaction centre is considered to be 'closed'. Depending on the prevailing conditions such as light intensity or temperature (which affects the metabolic state), a greater or lesser proportion of reaction centres may be closed. Closure will inevitably cause a decline in quantum efficiency of PSII.

Following the initial rise in fluorescence after the application of actinic light, the fluorescence signal then declines over a period of minutes, which is termed 'quenching' (Krause and Weis, 1991). Quenching of the fluorescence signal can arise from a combination of processes: first there is

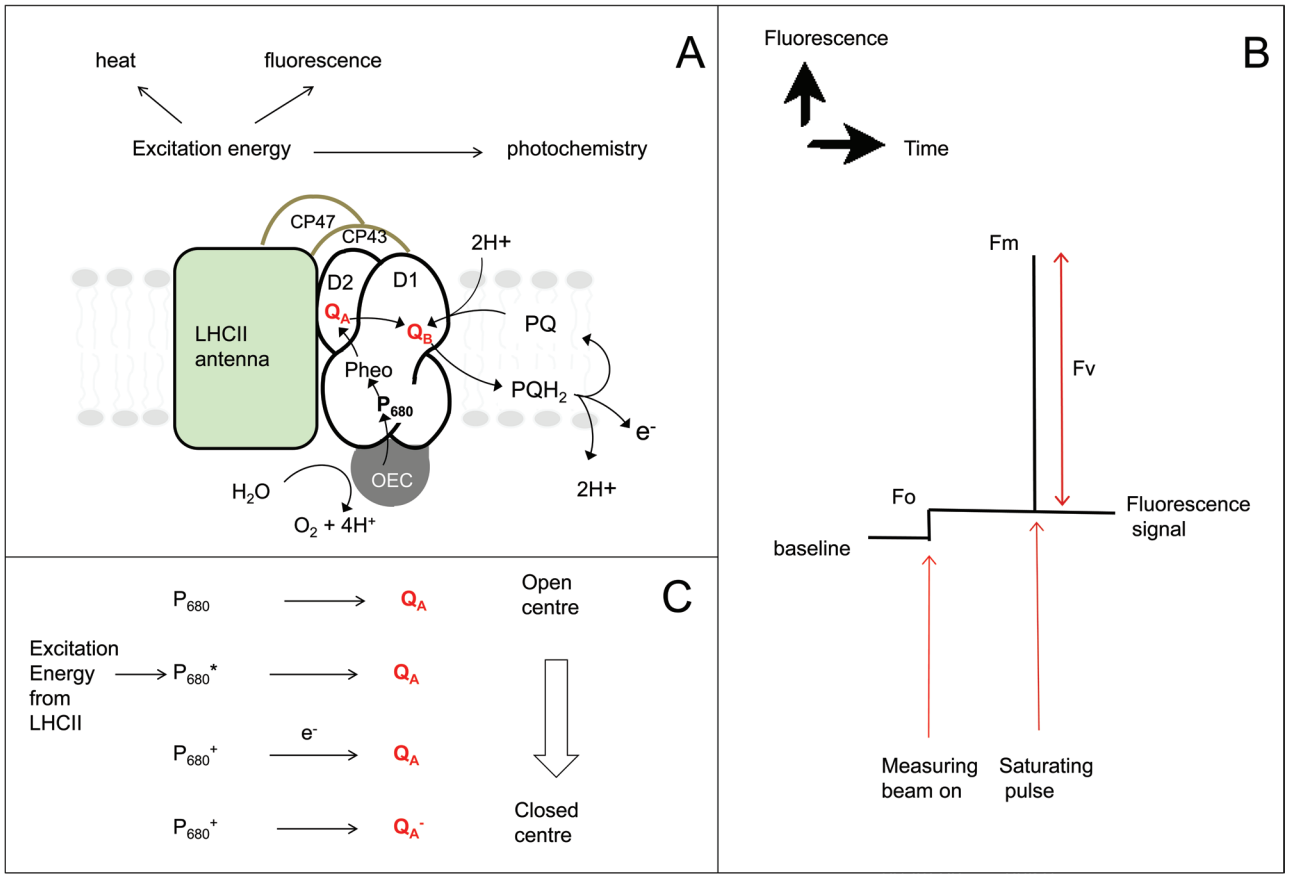


**Fig. 1.** A simplified depiction of events in PSII that lead to identification of key parameters in fluorescence analysis. (A) A schematic figure showing electron transport within the PSII reaction centre complex. Energy absorbed by chlorophyll within the light-harvesting complex can be dissipated via photochemistry, by heat (non-photochemical quenching), or as fluorescence. The competition between these processes allows us to resolve the efficiency of PSII. (B) A typical fluorescence trace made on dark-adapted leaf material showing how $F_o$ and $F_m$ are formed. The measuring beam excites chlorophyll but is not of a sufficient intensity to induce electron transport through PSII (i.e. 'charge separation' when Pheo is reduced). This gives $F_o$, the minimal level of fluorescence, and reaction centres are said to be open. A brief saturating pulse of light results in the formation of the maximum possible yield of fluorescence, $F_m$. During this pulse reaction centres are effectively closed. (C) A schematic figure explaining the transfer of energy and electrons within PSII that result in open and closed centres and the formation of $F_o$ and $F_m$ states, respectively. The excited state $P_{680}^*$ and subsequent transfer of an electron to the primary acceptor $Q_A$ gives rise to a closed centre. $Q_A^-$ cannot accept another electron until it has passed its electron onto the next electron acceptor, $Q_B$.

the light activation of the process of photosynthesis itself. In particular, key enzymes in the Calvin cycle require activation in order to achieve full activity (Buchanan and Balmer, 2005), and metabolite pool sizes in the stroma and cytosol need to increase. From darkness at ambient temperatures, this process typically can take several minutes or longer, with a large amount of variation caused by species and environment. Another factor is the opening of stomata, which increases the availability of $CO_2$ for Rubisco. Stomata tend to open and close an order of magnitude more slowly than photosynthetic events (Lawson *et al.*, 2012). All of these processes provide an increased availability of sinks for the electrons derived from the light-dependent processes in the thylakoid and contribute to the quenching by the process of photosynthesis itself, and this is termed photochemical quenching.

Secondly, on illumination, there is a rapid increase in the rate constant for heat dissipation of chlorophyll excitation energy, measured using a parameter termed non-photochemical quenching (NPQ). This is a photoprotective process that removes excess excitation energy within chlorophyll-containing complexes and prevents the likelihood of formation of damaging free radicals. This type of quenching competes with both fluorescence and photochemical quenching, and acts as a 'safe' mechanism for dissipating substantial levels of chlorophyll excitation energy, depending on the prevailing conditions and species (Demmig-Adams and Adams, 2006). The process of NPQ is regulated by the acidification of the thylakoid lumen due to the accumulation of protons in the thylakoid lumen (during linear and cyclic electron flow) that form a ΔpH (Horton *et al.*, 1996, 2008; Ruban *et al.*, 2012). It also involves the regulatory protein PsbS and the conversion of violaxanthin to zeaxanthin in the xanthophyll cycle (Li *et al.*, 2002; Kiss *et al.*, 2008; Murchie and Niyogi, 2011). Xanthophylls are carotenoids that play a critical part in NPQ (Demmig

Adams, 1990): the conversion of violaxanthin to zeaxanthin takes place in the light following the activation of the enzyme violaxanthin de-epoxidase by the acidification of the thylakoid lumen (Yamamoto *et al.*, 1999). Both protonation of PsbS and the formation of zeaxanthin result in conformational changes in PSII antenna that induce quenching of excitation energy in the antenna of PSII (Ruban *et al.*, 2012).

An overarching concept is that chlorophyll fluorescence analysis allows us to separate the components that make up all energy dissipation of chlorophyll in the thylakoid membrane. Early techniques for doing this involved applying chemicals such as herbicides that block electron transport in PSII (Krause *et al.*, 1982; Horton and Hague, 1988) to remove photochemical quenching, thereby giving an indication of what photochemistry was before the application of the herbicide. Clearly this is not practical on a routine basis, and techniques were developed that involved the application of a brief (<1 s) very bright saturating flash of light that temporarily closes all PSII reaction centres for the duration of its application (Bradbury and Baker, 1981; Quick and Horton, 1984). During the flash, the reaction centres are closed (see Fig. 1) and so the level of photochemical quenching is effectively zero and only non-photochemical processes will remain. The fundamental principle of the saturating flash/pulse is that fluorescence rises to a level corresponding to that which would exist without any photochemical quenching.

## Calculation of commonly used fluorescence parameters

Figure 2 shows a 'typical' fluorescence trace of the type that are repeatedly used to simplify the principles of chlorophyll fluorescence and provide an explanation of how we can derive important information about the performance of PSII by comparing fluorescence levels whilst exposing the leaf to a combination of actinic lights (i.e. light that drives photochemistry and photosynthesis), darkness, and a series of saturating pulses. This is based around the principle of separating the photochemical and non-photochemical components.

In the dark-adapted state (when all PSII centres are open and no NPQ is present), a 'measuring light' is switched on (Figs 1, 2). This light is of an intensity too low to induce electron transport through PSII but high enough to elicit a minimum value for chlorophyll fluorescence, termed $F_o$. The measurement of $F_o$ and its light-adapted equivalent $F_o'$ is fundamental to fluorescence analysis, and attention should be paid to the issues raised and recommendations in this review. $F_o'$ is measured immediately after switching off the actinic light, but accurate measurement is difficult: many instruments have the capacity to apply a weak far-red (FR) light to measure both $F_o$ and $F_o'$ (typically a few seconds). FR light stimulates PSI, drawing electrons from PSII to ensure that $Q_A$ remains fully oxidized during measurements. This is important since $Q_A$ can be partially reduced

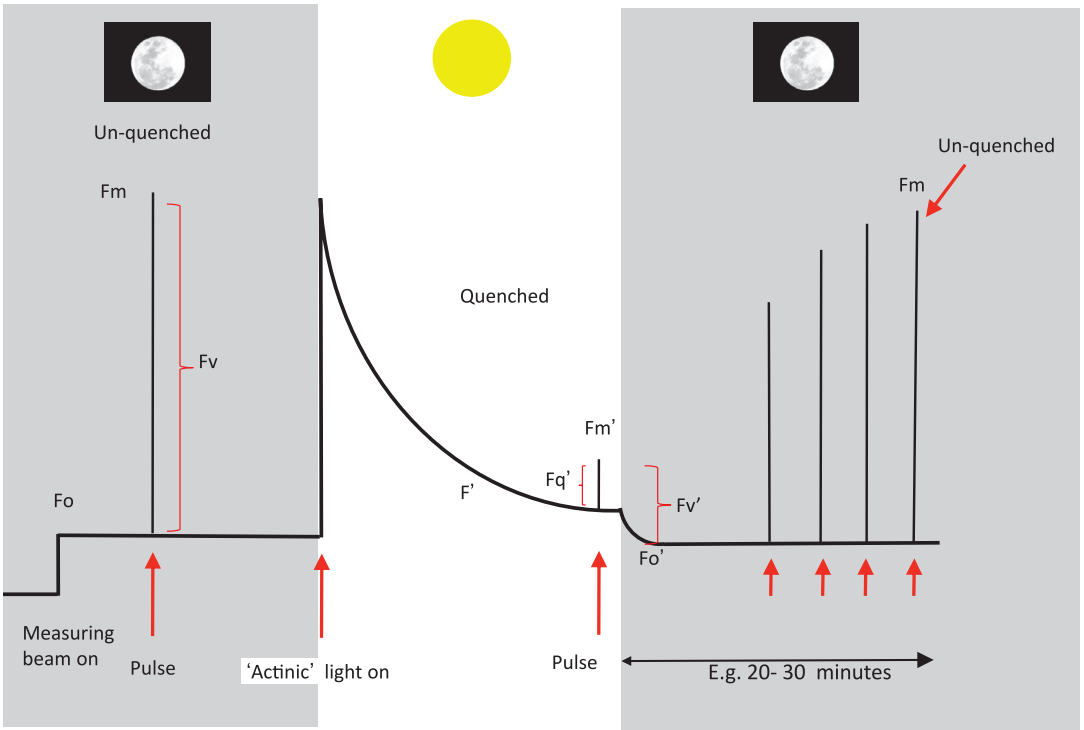


**Fig. 2.** A stylized fluorescence trace of a typical experiment using dark-adapted leaf material to measure photochemical and non-photochemical parameters. This would be typical of an induction at high irradiance of ≥500 μmol $m^{-2}$ $s^{-1}$. A true 'Kautsky' effect would be measured at moderate illumination, for example <200 μmol $m^{-2}$ $s^{-1}$, where transients corresponding to induction of photosynthesis are revealed. Note that the 'decay' of $F_o'$ in the dark after switching off the actinic light would be accelerated by adding FR light to stimulate PSI activity. More explanation is given in the text.

in the dark by chlororespiration (see review by Peltier and Cournac, 2002). An alternative approach is to calculate $F_o'$: see below.

Application of a saturating pulse to a dark-adapted leaf induces a maximum value of fluorescence by closing reaction centres. At this point, in a healthy non-stressed plant there is no NPQ because the material has been fully dark adapted; therefore, the maximal possible value for fluorescence, $F_m$, is recorded. The difference between $F_o$ and $F_m$ is the variable fluorescence, $F_v$. It has been shown theoretically and empirically that $F_v/F_m$ gives a robust indicator of the maximum quantum yield of PSII chemistry (Butler, 1978; Genty *et al.*, 1992). For unstressed leaves, the value of $F_v/F_m$ is highly consistent, with values of ~0.83, and correlates to the maximum quantum yield of photosynthesis (Demmig and Björkman, 1987). The existence of any type of 'stress' that results in inactivation damage of PSII (often referred to as photoinhibition, see below) (Long *et al.*, 1994) or the induction of sustained quenching (Demmig-Adams and Adams, 2006) results in a lowering of $F_v/F_m$. This has meant that measuring $F_v/F_m$ following an appropriate period of dark adaptation has been used as one of the most common techniques for measuring 'stress' in leaves.

If a sufficiently strong actinic light is now applied, we see an initial rise in fluorescence. This is then quenched as a result of the increasing competition with photochemical and non-photochemical events. The steady-state level of fluorescence in the light is termed, $F'$. Generally speaking, it may take several minutes or much longer (e.g. >20 min) to achieve a steady state from a dark-adapted state. The application of a saturating pulse under actinic illumination transiently closes all the reaction centres and provides a value of maximal fluorescence in the light-adapted state, termed $F_m'$. Note that this is less than the dark-adapted $F_m$ value due to the contribution of NPQ.

If we have both dark-adapted and light-adapted measurements we can measure three key parameters.

(i) The operating efficiency of PSII photochemistry, $F_q'/F_m'$ (Genty *et al.*, 1989), is calculated from $(F_m'–F')/F_m'$ (and does not require a dark-adapted measurement). This parameter is often termed ϕPSII or $\Delta F/F_m'$ (Maxwell and Johnson, 2000; Baker, 2008). It gives the proportion of absorbed light that is actually used in PSII photochemistry (Genty *et al.*, 1992) and can therefore be used to estimate the rate of electron transport through PSII with knowledge of light absorptance by the leaf and photosystems (which has technical difficulties associated with it, that are discussed below). Due to its simplicity of measurement $F_q'/F_m'$ is the most commonly measured and used light-adapted parameter. $F_q'/F_m'$ can be broken down into two products, the level of photochemical quenching of PSII ($F_q'/F_v'$) and PSII maximum efficiency ($F_v'/F_m'$) (a parameter that describes the maximum operating efficiency in the light-adapted state, with any decrease in this parameter reflecting an increase in NPQ).
(ii) The level of photochemical quenching of PSII, $F_q'/F_v'$ (often referred to as qP: Table 1) gives an indication of the proportion of reaction centres that are open. Some studies have used $1–F_q'/F_v'$ (or 1–qP) to denote the proportion of closed reaction centres (Huner *et al.*, 1996, 1998). It has also been used to indicate the onset of photoinhibition (Anderson *et al.*, 1995) and in determining the level of photoprotective quenching of fluorescence (Ruban and Murchie, 2012). The relationship between $F_q'/F_v'$ and the fraction of open PSII reaction centres is not considered linear, so such interpretations should be made with care. The parameter qL $[(F_q'/F_v')/(F_o'/F')]$ is considered a more accurate indicator of the PSII redox state (see Kramer *et al.*, 2004; Baker, 2008).

**Table 1.** *List of commonly used abbreviations and equations*

Note that this is included to identify only the most common parameters, and we refer the reader to comprehensive reviews of Baker (2008) and Maxwell and Johnson (2000).

| Parameter | Also known as | Formula (see Fig. 2) | Description |
|---|---|---|---|
| $F_v/F_m$ | | $(F_m–F_o)/F_m$ | Maximum quantum efficiency of PSII photochemistry. |
| $F_v'/F_m'$ | | $(F_m'–F_o')/F_m'$ | Maximum efficiency of PSII photochemistry in the light, if all centres were open. |
| $F_q'/F_m'$ | ϕPSII, $\Delta F/F_m'$ | $(F_m'–F')/F_m'$ | PSII operating efficiency: the quantum efficiency of PSII electron transport in the light. |
| $F_q'/F_v'$ | qP | $(F_m'–F')/(F_m'–F_o')$ | Photochemical quenching: relates PSII maximum efficiency to operating efficiency. Non-linearly related to proportion of PSII centres that are open. See qL. |
| NPQ | | $(F_m–F_m')/F_m'$ | Non-photochemical quenching: estimates the rate constant for heat loss from PSII. |
| qL | | $(F_q'/F_v')/(F_o'/F')$ | Estimates the fraction of open PSII centres. |

(iii) The level of NPQ (see Table 1) is the only measurement that requires knowledge of the dark-adapted values of $F_o$ and $F_m$. Therefore, if the dark-adapted $F_v/F_m$ value is substantially lower than 0.83, this value should be treated with caution and in particular NPQ in leaves with differing $F_v/F_m$ values should not be compared.

## To dark adapt or not?

A key question asked by many researchers is always 'for how long should I dark-adapt my leaf?'. To answer this, the user must appreciate that NPQ contains different components as we explain below (Walters and Horton, 1991; Horton *et al.*, 1996). Switching off the actinic light allows us to re-measure the minimal level of fluorescence, $F_o'$. To obtain this accurately in a light-adapted leaf, we need to ensure that PSII is fully oxidized, and this can be achieved using a pulse of FR light. Figure 1 demonstrates that by applying regular saturating pulses we can follow the relaxation of $F_m'$ in the dark until it reaches its dark-adapted value again.

For advanced users, the kinetics of relaxation allows us to separate NPQ into different components that relax slowly or more quickly, depending on their origin. However, separation of these components is not trivial in the field (Murchie and Horton, 2007). The majority of NPQ is usually made up of high energy state quenching or qE that occurs via the mechanisms described above (Walters and Horton, 1991; Murchie and Horton, 2007). Two other components exist: quenching caused by state transitions (qT) which refers to the migration of peripheral LHCIIs from PSII to PSI. This is normally associated with low light conditions and its significance in high light is thought to be small. Second photoinhibitory quenching (qI) is small at moderate and low light but becomes important at high saturating light levels. qI is often used to refer to any sustained quenching (i.e. is not removed by dark adaptation). Photoinhibition is usually assumed to arise from inactivation of PSII by photooxidation followed by a repair cycle, whereby PSII is partially disassembled and the damaged D1 protein is repaired by *de novo* synthesis and reassembled (Yokthongwattana and Melis, 2006; Sun *et al.*, 2010). Long-lived quenching is also associated with the accumulation and retention of zeaxanthin (Demmig Adams *et al.*, 2006; Horton *et al.*, 2008). A rise in $F_o$ is associated with photoinhibitory damage but not with zeaxanthin retention.

Note that it is possible to use the changes in $F_v'/F_m'$ as an indicator of changes in NPQ as the two parameters will coincide non-linearly, but note also that it can be inaccurate at higher values of NPQ (e.g. Murchie *et al.*, 1999). More accurately, changes in $F_v'/F_m'$ give an indication of the contribution NPQ makes to $F_q'/F_m'$.

## Pitfalls and good practice for the fluorescence user

In practice, it is rarely possible or necessary to go through an entire dark–light–dark protocol as shown in Fig. 2. For example, in the field, it is common due to time constraints to perform one measurement within one saturating pulse with or without a dark adaptation period, in which case the user will need to extract as much useful information as possible from a limited set of data. The following section should help the user select the correct protocol to maximize the information obtained from their experiment and avoid some common pitfalls. To supplement this section, we have included several protocols at the end of this review that, if followed, would help the novice to understand the issues.

### *Issues with the dark-adapted state*

One of the first questions that faces a user is 'should I dark adapt? Clearly this depends on the objectives of the experiment and the time and equipment available. There is sometimes an assumption that dark adaptation is necessary to achieve the best results, but it must be decided first whether information on photoinhibition or sustained down-regulation of PSII is required or whether operational photosynthesis in the growth conditions is more important.

Returning the leaf to a fully dark-adapted state ($F_v/F_m$ value of ~0.83) is essential for studies of NPQ and for quenching analysis, if that is what is required. Some users prefer to use pre-dawn $F_v/F_m$ values, and this is advisable if the experiment involves persistent, severe long-term suboptimal conditions such as drought. The presence of low pre-dawn $F_v/F_m$ will indicate substantial photoinhibition or down-regulation of PSII. A good example would be the sustained dissipation of reaction centres in over-wintering evergreen plants (Demmig Adams and Adams, 2006) or severe drought stress (Flexas *et al.*, 2002). Note that mild drought associated with stomatal closure will not by itself usually result in a substantial decline in $F_v/F_m$ (for a review, see Baker and Rosenqvist, 2004). Diurnal photoinhibition where the $F_v/F_m$ values recover more rapidly is common in many environments (Ogren, 1988; Demmig-Adams *et al.*, 1989; Murchie *et al.*, 1999). The best answer to this question is that it should be tested on the species under study using non-stressed control conditions by dark adaptation from a steady-state condition in the light and then frequent measurement every 5–10 min until a steady value of $F_v/F_m$ is reached. In practice, if the plant is well adapted to its environment and temperatures are within an optimal range, 20–30 min should be sufficient to remove all qE and qT components. Any remaining sustained quenching is usually referred to as qI and, as mentioned, can be caused by photoinactivation (damage) to the D1 protein or a long-lived quenching state caused by retention of zeaxanthin. Inactivation of and damage to D1 results in an increase in $F_o$, but long-lived zeaxanthin quenching does not. The difficulties associated with measuring $F_o$ and $F_o'$ are covered below.

Here we deal with the measurements of $F_o$ and $F_m$ in the dark. Accurate measurements of $F_o$ in the field are made difficult due to relaxation kinetics of the return to the dark-adapted state. When a leaf is placed in the dark, the movement of electrons in the thylakoid should cease almost immediately. However, NPQ 'relaxes' more slowly because the protective non-photochemical quenching processes remain active (such as zeaxanthin). Therefore, to discover the

true maximum value of fluorescence, we need to allow the leaf to remain in the dark for a length of time sufficient for these processes to complete (i.e. NPQ to become zero). The length of this process depends on factors such as the state of de-epoxidation of the xanthophyll cycle; see above (Horton *et al.*, 1996). Some devices provide a pulse of FR light for measuring $F_o$ in a dark-adapted leaf to ensure that $Q_A$ is fully oxidized, and this should be used where available. This can also be an issue if $Q_A$ reduces due to reduction of plastoquinone by chlororespiration (Baker, 2008). Another pitfall at this state is avoiding charge separation in PSII; that is, inducing electron transport by using either a high measuring beam intensity or an overly high frequency of modulation. This can result in a false value of $F_o$. It is straightforward to adjust the measuring beam intensity: if it induces quenching or a rise in fluorescence when measuring a dark-adapted leaf, then consider lowering the intensity. During actinic or pulse application, it is necessary to have a high frequency of modulation to help reduce the effect of variation in the signal, but this is not recommended in the dark-adapted state. The frequency is often adjusted automatically by the device software during pulsing, but it is advisable to check the manufacturer's instructions.

The dark-adapted value of $F_v/F_m$ is commonly used to assess plant 'stress', and has proved invaluable in many studies (e.g. Maxwell *et al.*, 1994; Murchie *et al.*, 1999), reflecting the high sensitivity of PSII to environmental stimuli, either directly or indirectly. However, its interpretation is not always straightforward, for a number of reasons.

(i) First, the underlying cause of the decline cannot be assessed easily. The inactivation of PSII reaction centres leading to oxidative damage and removal of PSII reaction centres is usually associated with heat loss and reduction in $F_v/F_m$ (rise in $F_o$). Caution must be applied here, however, because the optical properties of the leaf can change under certain stress conditions such as drought, meaning that the individual values of $F_m$ and $F_o$ may be a result of changes in leaf absorptance. Examples of optical properties under drought can be shown by the change in reflectance spectra (e.g. Babar *et al.*, 2006). These errors are removed when the ratio $F_v/F_m$ only is considered.

(ii) It is often stated that a decline in $F_v/F_m$ is evidence that the photosynthetic performance of the plant is compromised. This is not necessarily the case: $F_v/F_m$ measures the intrinsic quantum yield of PSII, and as such it should correlate with the maximum quantum yield of photosynthetic gas exchange ($CO_2$ or $O_2$). However, the latter is measured by the linear portion of a light response curve (i.e. under low light-limiting conditions). It is therefore possible (and presumably relatively common) for a leaf to have a reduced $F_v/F_m$ but no penalty to photosynthesis in high and saturating light conditions (Demmig-Adams and Adams, 2006; Murchie and Niyogi, 2011). For leaves that have undergone severe light stress, a reduction in both light-limited and light-saturated photosynthesis is possible.

(iii) There may be a requirement for a sufficiently high light intensity to induce inactivation of PSII (Takahashi and Murata, 2008) before a difference in $F_v/F_m$ is observed between treatments.

(iv) $F_v/F_m$ assesses the maximum quantum yield of PSII only. A site of stress in another part of the plant (e.g. roots) may not manifest itself as a change in $F_v/F_m$. This may seem an obvious statement, but it is included to prevent the assumption that fluorescence is a generic 'plant stress detector'.

### *Problems with measurements in the light*

Due to its ease of measurement, $F_q'/F_m'$ (also known as ϕPSII) is a highly popular measurement and has largely established itself as an accurate indicator of operational PSII efficiency in the light (Genty *et al.*, 1992; Maxwell and Johnson, 2000; Baker, 2008). Some inaccuracies of the light pulse technique have been pointed out: for example, PSI can contribute to fluorescence when measurements are made above 700 nm (e.g. Genty *et al.*, 1990) and the existence of 'multiple turnovers' of PSII during the saturating pulse (Vernotte *et al.*, 1979). However, these are now considered to be mostly small or rare partly due to high level of agreement between $F_q'/F_m'$ and the quantum yield of $CO_2$ assimilation (Baker 2008).

In the field, $F_q'/F_m'$ is an extremely useful parameter because it is possible to derive rates of PSII electron transport (although several caveats to this are discussed below). However, consideration must first be given to the factors that cause the values to change. First, it is highly dependent on the ambient light levels and can vary substantially with even a relatively small change in photosynthetically active radiation (PAR). Therefore, making measurements in the natural environment, one should interpret the data with this in mind by incorporating equipment that accurately measures PAR at the leaf surface. Alternative techniques have restricted measurements to periods of the day where PAR is known to be saturating for photosynthesis and electron transport (Murchie *et al.*, 1999, 2002).

Since $F_q'/F_m'$ directly measures the quantum yield of PSII electron transport it is possible to calculate the rate of electron transport directly using knowledge of absorbed PAR and the fraction of light intercepted by PSII and PSI. Determining absorption by the two photosystems is extremely difficult to quantify accurately, and users often make the assumption of equal absorption by both PSII and PSI (using a value of 0.5). Absorbed PAR can be relatively accurately determined using an integrating sphere or similar device, and this is highly recommended, although this type of measurement tends to be rare. Instead a value of 0.84 is often used and considered to be an average value of leaf absorption. Although these 'standard values' are usually tacitly assumed to be 'constants', the user is warned against comparison of leaf samples that are likely to have differing optical properties that will affect light absorption (or the same leaf undergoing stress treatment). For example, comparing electron transport rate (ETR) values in a drought-stressed leaf with a low turgor value with a control hydrated leaf is not appropriate. Leaf samples with different pigment contents or photosystem stoichiometry such as those that have undergone changes in light acclimation state (Walters *et al.*, 2005; Anderson *et al.*, 1995) may also suffer inaccuracies.

### *Errors in measurements of $F_o'$*

The measurement of $F_o'$ can be open to error if the FR light applied does not adequately oxidize $Q_A$ and if the relaxation of NPQ causes $F_o'$ to increase rapidly after the actinic light has been switched off. A solution to this was presented by Oxborough and Baker (1997a) who suggested that $F_o'$ can be calculated from light-adapted measurements with knowledge of $F_o$ and $F_m$, utilizing the quenching of $F_m'$ using the following equation (Equation 1).

$$F_o' = F_o / \left[ (F_v / F_m) + F_o / F_m' \right] \quad (1)$$

## Measuring the redox state of PSI

It is becoming increasingly common to include measurements of the PSI redox state (Klughammer and Schrieber, 1994) and directly compare them with measurements of $F_q'/F_m'$, measured as above. Some devices combine these measurements for simultaneous measurement. Cyclic electron flow around PSI affects the accumulation of ΔpH and has a role in photoprotection and regulation of the 'redox poise' of the thylakoid electron transport chain (Nandha *et al.*, 2007; Johnson, 2011). As this has a role in determining PSII events, including NPQ, it is wise to assess briefly the role of these measurements here. This is achieved by monitoring the redox state of the reaction centre chlorophyll of PSI, P700, via absorbance in the near infrared (peak at 810–840 nm) (Klughammer and Schreiber, 1994). Combining ϕPSII and ϕPSI measurements can be valuable (Johnson, 2011). However, the same principles apply with measuring PSII ETR above: comparing PSII:PSI ETR ratios in contrasting leaves should be approached with great caution, and the difference between ETR of PSII and PSI cannot give the rate of cyclic electron transport without empirical correction for stoichiometry and light absorption.

## Different applications of chlorophyll fluorescence

### *Relationship to $CO_2$ assimilation*

In whole-leaf studies it is natural to extend the interpretation of chlorophyll fluorescence data to analyse its impact on photosynthetic rates of $CO_2$ assimilation and, by inference, productivity of the plant or system in question.

Under certain controlled circumstances, the $F_q'/F_m'$ measured by fluorescence is accurately correlated with rates of $CO_2$ assimilation which has added extra interest to extend the possibilities of this technique and has led to advances in our understanding of photosynthetic regulation (Genty *et al.*, 1989, 1990; Cornic, 1994). This relationship makes intuitive sense because the products of linear electron transport, ATP and NADPH, are used directly in photosynthetic carbon assimilation in known ratios. In $C_3$ plants, this close correlation can be observed best when photorespiration is inhibited by lowering the partial pressure of oxygen to 2%. Why? The electron requirement for assimilation of one $CO_2$ molecule in leaves where photorespiration is inhibited is four. This number will rise as the proportion of carbon flux through the photorespiratory pathway rises, for example as happens during stomatal closure (Wingler *et al.*, 1999; Flexas *et al.*, 2002). Therefore, whenever the electron requirement is altered, the relationship between $F_q'/F_m'$ and $CO_2$ assimilation rate is also altered. Unfortunately, for field-based measurements, the number of factors that can cause this is high: stomatal closure (as mentioned below), temperature (this changes the relative rates of carboxylation and oxygenation in the leaf), abiotic stress (this results in an increased activity of alternative electron sinks such as the Mehler reaction and cyclic electron transport), leaf development, and shading. A common example given of this disconnect between $F_q'/F_m'$ and the $CO_2$ assimilation rate is that of species in environments undergoing multiple stresses (Cheesman, 1991). In conclusion, this means that linearity is difficult to achieve unless tightly controlled conditions are used and care is taken when comparing samples in which the ratio of allocation between $CO_2$ assimilation and other processes is known not to have changed. The relationship in $C_4$ plants is much more easily achieved due to the suppression of photorespiration by the $CO_2$-concentrating mechanism. Another point of error is the one mentioned above regarding the accurate measurement of ETR.

### *Imaging of chlorophyll fluorescence*

Imaging of chlorophyll fluorescence is becoming increasingly popular as a screening (Barbagalo *et al.*, 2003) and diagnostic tool (Baker, 2008), due mostly to the development of instrumentation (Oxborough, 2004), with many commercial instruments available through companies such as Photon Systems Instruments (Brno, Czech Republic); Walz (Effeltrich, Germany), and Technologica Ltd (Essex, UK), amongst others. Chlorophyll fluorescence imaging has also recently been incorporated into many phenotyping platforms for high-throughout phenotypic analysis. There are several advantages to imaging, and below we have explored some of these along with some examples of uses.

#### *Screening*

Chlorophyll fluorescence imaging allows multiple plants to be monitored at the same time under identical conditions, providing an ideal screening platform. Chlorophyll fluorescence directly relates to the rate of energy flow via the electron transport chain and therefore any perturbation that impacts on plant metabolism (e.g. pathogen infection) will impact on fluorescence parameters even if not directly linked to photosynthesis (Barbagallo *et al.*, 2003). Examples of screening multiple plants include early detection of herbicide application (Barbagallo *et al.*, 2003; Jin *et al.*, 2011), nutrient deficiency (Mauromicale *et al.*, 2006), drought stress (Rahbarian *et al.*, 2011), identification of $CO_2$-sensitive photorespiratory *Arabidopsis thaliana* mutants (Badger *et al.*, 2009), and plants with improved photosynthesis and crop yield (Baker and Rosenqvist, 2004; Chaerle *et al.*, 2007; Baker, 2008; Harbinson *et al.*, 2012).

*Spatial and temporal heterogeneity*

An advantage of any imaging technique is the detailed spatial representation of the measured parameter, allowing the assessment of sample heterogeneity. Monitoring changes within such images provides additional temporal assessment of the measured parameter. Examples of spatial heterogeneity highlight the value and appeal of imaging, by drawing attention to the fact that 'detection of symptoms', or assessment of 'reductions in a specific fluorescence parameter' may well have been missed if a traditional fibre optic approach had been utilized, which is only capable of measuring a small area/proportion of the leaf. There are numerous examples of chlorophyll fluorescence imaging for detecting within-leaf or plant treatment effects, including the assessment of freezing tolerance and cold acclimation in *Arabidopsis* (e.g. Ehlert and Hincha, 2008), insect herbivory (e.g. Tang *et al.*, 2006), leaf fungal infection (e.g. Scholes and Rolfe, 2009; McElrone *et al.*, 2010), and the impact of ozone damage (Leipner *et al.*, 2001; Aldea *et al.*, 2006).

*Degrees of resolution*

Depending on instrumentation, images of chlorophyll fluorescence can be obtained at a range of resolutions. High-resolution images of stomatal guard cells have been used to determine efficiency and quenching parameters from individual chloroplasts (Baker *et al.*, 2001; Fig. 3). The spread of disease and host–pathogen interactions has been monitored both spatially and temporarily at the leaf level using high-resolution images of NPQ and $F_q'/F_m'$ (Scholes and Rolfe, 2008), while Aldea *et al.* (2006) illustrated fine-grain heterogeneity in mapping the spatial patterns of the production of reactive oxygen species (ROS) on PSII quantum efficiency following viral infection or ozone damage. A high-resolution microscope imaging system was used to show the impact of water stress and $CO_2$ concentration on guard cell photosynthetic efficiency in a range of different plant types by Lawson *et al.* (2003). A similar system has been used to detect the impact of heavy metals such as cadmium on the aquatic plant *Lemna* (Fig. 3) Three-dimensional chlorophyll fluorescence imaging has also been used in the detection of herbicide effects on a whole plant (Eguchi *et al.*, 2008).

*Combined with other techniques*

The combination of chlorophyll fluorescence imaging with other measurement techniques and instrumentation can provide a unique research tool, that enables users to answer novel questions. For example, using chlorophyll fluorescence imaging with infra-red gas exchange (IRGA) techniques enables the user to correlate PSII photosynthetic efficiency directly to the IRGA-measured $CO_2$ assimilation rate by eliminating photorespiration through the reduction of $[O_2]$ or increase in $[CO_2]$ within the chamber. For example, such calibration techniques have been instrumental in developing protocols and procedures to visualize patterns of $CO_2$ diffusion in leaves (Morison *et al.*, 2005; Lawson and Morison, 2006), enabling the determination of gas fluxes within leaves of different species (Morison *et al.*, 2007), taking into account the venation patterns of the leaf (Lawson and Morison, 2006) and the importance of fluxes on carbon gain (Pieruschka *et al.*, 2005, 2006; Morison and Lawson, 2007). Control of the gas environment around the samples being imaged enables users to identify specific plants traits; for example, photorespiratory mutants were identified under a zero $[CO_2]$ environment, with plants maintained in an air-tight box with a layer of $CO_2$-absorbing material on the bottom (Badger *et al.*, 2009). Using a high-resolution microscope chlorophyll fluorescence imaging system, Lawson *et al.* (2002) employed a specially designed IRGA chamber to control $[CO_2]$ and $[O_2]$ as well as humidity, to show for the first time in intact green leaves that Calvin cycle activity was the major sink for guard cell photosynthetic electron transport. Using humidity as a driver of stomatal behaviour (which does not directly affect photosynthesis), the same authors also showed that the opening and closing of stomata is not linked to guard cell photosynthetic efficiency unless the closure reduces the internal $CO_2$ concentration to which guard cell photosynthetic electron transport responded (Lawson *et al.* 2002). The combination of chlorophyll fluorescence imaging with other imaging technologies is also proving an extremely powerful tool in the development of large-scale phenotyping protocols and platforms. Although a full description of such phenotype imaging is beyond the scope of this review, the use of combined chlorophyll fluorescence imaging and thermography can supply critical information on photosynthetic rates in relation to stomatal behaviour (Chaerle *et al.*, 2007) and, if performed under controlled conditions with appropriate calibrations, could provide an approach for imaging intrinsic water use efficiency (iWUE) (Lawson, 2009). Chlorophyll fluorescence imaging has also been combined with hyperspectral imaging for early detection of head blight disease in wheat (Bauriegel *et al.*, 2011). The advantage of this combined system enables chlorophyll degradation and the impact of different diseases to be distinguished based on changes in photosynthetic efficiency and spectral signatures such as those used in remote sensing that assess vegetation status, such as the Normalized Difference Vegetation Index (NDVI).

There are also a couple of disadvantages to imaging chlorophyll fluorescence relative to the use of a standard fibre optic system. The cost of the instruments tends to be greater than those of fibre optic fluorimeters (although inexpensive compared with buying several fluorimeters for measuring several plants simultaneously). Chlorophyll fluorescence imaging systems tend to require large banks of light-emitting diodes (LEDs) to ensure even actinic illumination and fully saturating pulses over the entire imaging area. This makes the instruments relatively large in size, and, in general, they are considered laboratory instruments and not portable systems, such as many of the hand-held devices. Having said this, several manufacturers have released field-based hand-held imaging systems (many of which are combined with IRGA instruments). For example, Walz extended its *M-Series* Imaging-PAM to include a MINI version for application in the field. This is a compact design, with high magnification and resolution, and can be mounted on their standard gas exchange equipment for dual measurements (http://www.walz.com/products/chl_p700/imaging-pam_ms/mini_version.

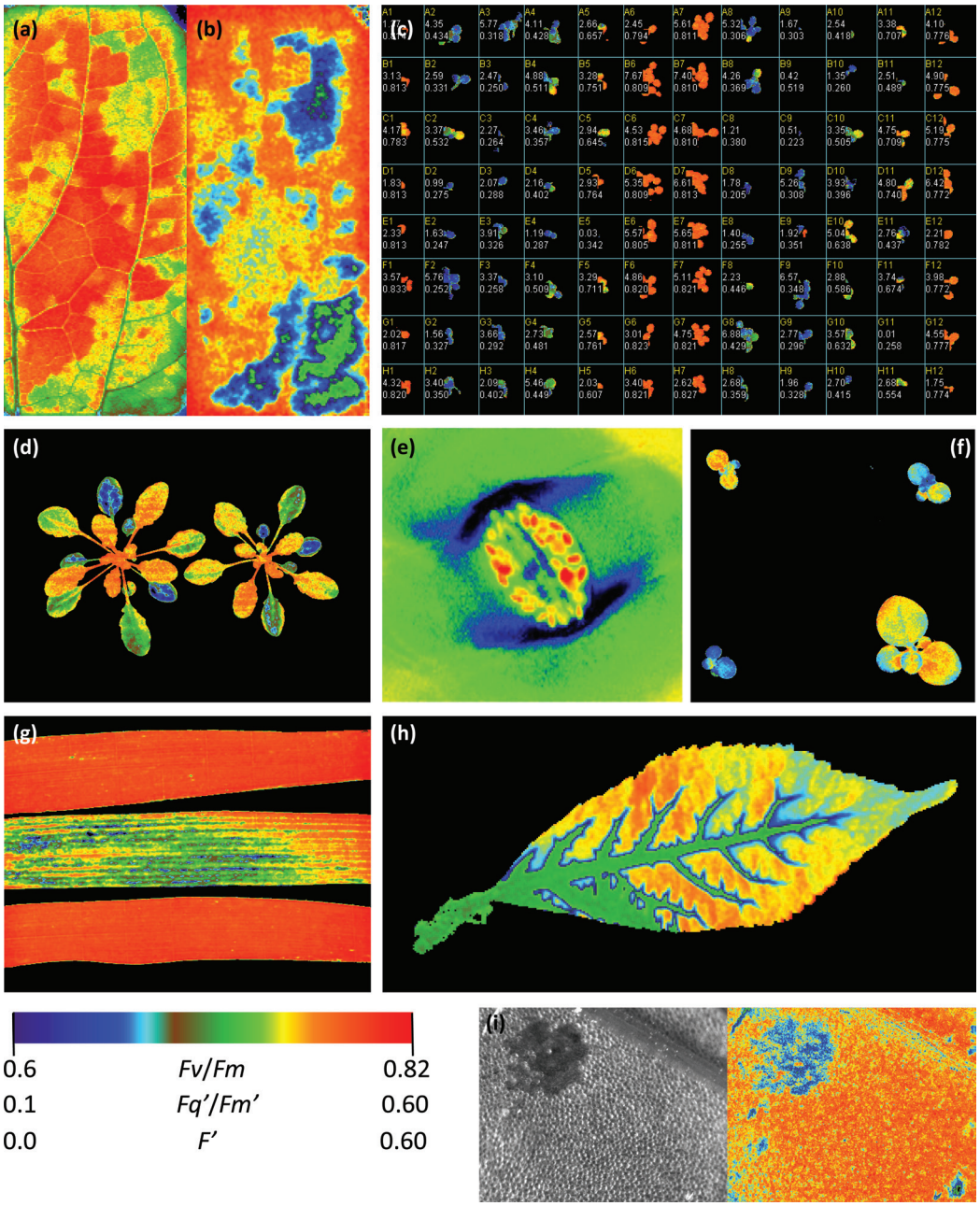


**Fig. 3.** (a) Images of steady-state fluorescence (*F*′) and (b) photosynthetic efficiency of PSII photochemistry ($F_q'/F_m'$) from 10 cm$^2$ leaf area enclosed in a chamber during simultaneous gas exchange measurements demonstrating the effect of patchy stomatal closure. Images were captured 5 min after dropping the humidity rapidly from 80% to 20%. All other chamber conditions were maintained at 200 μmol m$^{-2}$ s$^{-1}$ PPFD, 400 mmol mol$^{-1}$ [$CO_2$], 2% [$O_2$], and a temperature of 24 °C. (c) An example of chlorophyll fluorescence as a screening technique showing the detection of the effects of herbicide treatment on $F_v/F_m$ of 1-week-old *Arabidopsis* plants growing on a 96-well plate (unpublished image of Technologica Ltd, with permission). (d) An $F_q'/F_m'$ image showing the heterogeneity of two *Arabidopsis* plants after 10 min acclimation to 300 μmol m$^{-2}$ s$^{-1}$ PPFD following induction of heterogeneity with 1 h at a high light intensity of 1000 μmol m$^{-2}$ s$^{-1}$. (e) An image of $F_v/F_m$ of the epidermis of an intact *Tradescantia* leaf showing the guard cell chloroplasts following the methods of Oxborough and Baker (1997b) taken with a high resolution microscope chlorophyll fluorescence imaging system (unpublished image of McAusland and Lawson). (f) Use of the chlorophyll fluorescence parameter $F_q'/F_m'$ to screen transgenic plants that have reduced levels of SBPase. The plant in the upper right and lower left have much greater reductions in SBPase than the other two plants. (g) Images of $F_v/F_m$ taken after 20 min dark adaption from three different varieties of wheat plants grown in the field, with different susceptibilities to rust. The middle images show a variety that is more susceptible to rust (unpublished image of Lawson and Driever). (h) A cut elder leaf illustrates the decrease in photosynthetic efficiency ($F_q'/F_m'$) with DCMU uptake via the petiole during transpiration. (i) High-resolution image of the effect of Cd on photosynthetic efficiency in the aquatic plant *Lemna*. The image was captured after stabilizing to 200 μmol m$^{-2}$ s$^{-1}$ light after having been exposed to Cd for 1 d. The image on the left represent steady-state fluorescence (*F*′) while that on the right is a false-colour image of $F_q'/F_m'$. (Unless stated all images are unpublished data of TL.)

html). Photon Systems Instruments (http://www.psi.cz/), also produce a number of portable leaf chamber imaging platforms, such as 'FluorCam' that is designed to attach to a range of standard gas exchange chambers produced by a number of commercial manufacturers. Customized fluorescence imaging systems are also available for large-scale scanning in the field, in which the lights and camera are enclosed in a cabinet (for dark-adapted measurements) and the entire system is on wheels (http://qubitsystems.com/plant-and-soil/z240-rover-fluorcam/), allowing it to move over crops collecting images. Large-scale measurements of chlorophyll fluorescence parameters are desirable and currently of great interest to researchers who use unmanned aerial vehicle (UAV)-based platforms for remote sensing (e.g. Zarco-Tejada *et al.*, 2011); however, a discussion of these systems is beyond the scope of this review.

Examination of the literature illustrates many examples of uses of chlorophyll fluorescence imaging either on its own or in combination with other instruments; however, due to space restrictions we have selected and illustrated only a of selection of them.

The automation and incorporation of in-built algorithms in many commercial fluorimeters has led to an increase in their use. However, as with any technique, to obtain robust and meaningful results, a suitable protocol must be designed first (Scholes and Rolfe, 2009). The requirements for imaging are identical to those described above for use with any fluorometer. However, below we have outlined additional requirements that need to be followed in order to obtain meaningful results. As imaging is working on a relatively large illuminated surface, it is critical that the leaf or material of interest is held horizontal to the actinic and modulated lights in order to prevent heterogeneous illumination over different areas of the leaf. The material should also be held at the correct height relative to the light and camera in instruments that rely on in-built light calibrations (based on a distance) and do not provide a measurement of PAR at the measurement surface. Two elements are important for imaging chlorophyll fluorescence: the light source must provide even illumination over the entire surface of the imaged sample; and the light source must be capable of providing a saturating pulse over the entire imaging area that is of sufficient intensity to close the majority of PSII centres and provide a representative $F_m'$. Saturating pulses of ~2000 μmol $m^{-2}$ $s^{-1}$ are probably sufficient for dark-adapted $F_m$ but may well be too low to provide a true $F_m'$ under high actinic illumination. Most commercial instruments provide between 4000 and 8000 μmol $m^{-2}$ $s^{-1}$. If you are working with $C_4$ plants, you may need higher saturating pulses. Before purchasing an instrument, it is worthwhile determining the instrument's capabilities in terms of the intensity of the saturating pulse and how even the illumination is over the sampling area. Another difference between imaging systems and fibre optic systems is how $F_o'$ is determined, the value of which is required to calculate quenching parameters. Most fibre optic system incorporate LEDs that provide an FR pulse (see above). However, many commercial imaging systems rely on banks or panels of LEDs that provide the measuring beam and the actinic source, and they tend to be on one waveband and are therefore not capable of carrying out an FR pulse. Therefore, many imaging system calculate $F_o'$ using the mathematical algorithm developed by Oxborough and Baker (1997a) (Equation 1)

The use of this method to estimate $F_o'$ in situations where plants are stressed and may experience significant photoinhibition has been queried (Maxwell and Johnson, 2000). However, this is not valid, as the only requirements for the calculation of $F_o'$ to be accurate are: (i) that PSII centres are open at the point of measuring $F_o$; (ii) that there is no reversal of down-regulation between the measurements of $F_o$ and $F_m$; and (iii) that there is no reversal of photoinhibition between the measurements of $F_m'$ and $F_m$ (for further information, see Lawson *et al.*, 2002; Oxborough, 2004). It is been argued that the calculation of $F_o'$ is actually more accurate than the measured value, due to the difficulty in measuring $F_o'$ (Lawson *et al.*, 2002).

## Techniques for long-term and remote monitoring of chlorophyll fluorescence

The field physiologist will usually need to relate measurements made over short time scales (seconds/minutes) to effects on growth and development that occur over weeks and months, which emphasizes the need for good experimental design. This is important for measurements of primary productivity. If we consider that the measurement of radiation use efficiency (RUE) of a plant canopy is weight of dry matter per unit radiation absorbed by the canopy, then to understand how cumulative short-term changes in photosynthesis, for example caused by mid-day depression of photosynthesis or canopy development, impact RUE we will need to quantify these processes on a daily basis (Black *et al.*, 1995; Murchie *et al.*, 1999). 'Spot' fluorescence measurements using hand-held equipment are quick to take but require high sampling frequency in order to integrate with environment flux, and it is difficult to take enough measurements to cover such growth periods or season-long periods, especially in inhospitable conditions.

Limitations of single instruments can be caused by power supply and low numbers of measuring sensors. However, there are now devices available on the market that have been designed for long-term monitoring of fluorescence using the modulated technique described above, for example the Walz Monitoring PAM (Porcar-Castell *et al.*, 2008). The design is modular, with a series of measuring heads, each containing the optics and light sources required for measurement of key parameters and connected physically to a data collection unit or a computer. It is possible to power the entire system using solar panels and upload the data remotely, requiring no user interaction except in cases where the leaf becomes dislodged. In practice, several measuring heads are deployed in vegetation and fixed to a leaf where they will remain until the user moves them. The head is capable of measuring $F'$ and, by applying saturating pulses, $F_m'$ as

programmed. During the night period, the values of $F_m'$ and $F_o'$ become $F_m$ and $F_o$, respectively. Although this system is limited by the inability to measure $F_o'$ effectively, it has already proved useful in studies assessing the annual variation in NPQ in a boreal forest (Porcar-Castell, 2011) and assessing changes in NPQ during development of rice canopies (E. Murchie, unpublished data). With development of systems that are less expensive and easier to deploy, this approach should prove invaluable to the field physiologist looking for 'high-resolution' data, especially where data can be uploaded remotely.

Other approaches have analysed the remote sensing of 'passive' fluorescence by satellite or by platform, exploiting the gaps in the solar spectrum—Fraunhoffer lines—which correspond to the wavelengths at which emission of chlorophyll fluorescence occurs (Moya *et al.*, 2004; Meroni *et al.*, 2009). This is promising for some applications and offers the unique ability to assess photosynthesis continuously over large areas, although it clearly has severe limitations in comparison with the accuracy and precision of leaf-level measurements. Nevertheless, correlation of key drought-related parameters with chlorophyll fluorescence signal could be obtained using a UAV (Zarco-Tejada *et al.*, 2012).

Such passive techniques have the disadvantage of uneven illumination of the field of view and no saturating pulse. Laser-induced fluorescence transient (LIFT) is a technique that uses a laser to project an excitation beam (e.g. 100 mm diameter in current devices) onto a target area of canopy. Fluorescence emission is collected using a telescope, filtered, and detected using a large photodiode. Data are processed in real time for an immediate, remote measurement of PSII yield and electron transport. This technique compares well with that made by routine fluorometry, and so far it has been used successfully for ecophysiological studies and remote sensing from distances of 5–50 m or more (Ananyev *et al.*, 2005; Pieruschka *et al.*, 2008, 2012; Malenovsky *et al.*, 2009) and can provide spatial distribution of photosynthetic efficiency. This is a bespoke system, but LIFT hardware, protocols for fluorescence induction, fluorescence data analysis, sensing, and data processing are described in the literature (e.g. Ananyev *et al.*, 2005).

## Concluding remarks

This article provides an update on previous reviews on the technique of chlorophyll fluorescence. Inspired by the new applications of this technique in crop phenotyping and monitoring, we have covered the aspects of methodology the user should be aware of, along with some new and emerging variations.

This technique lends itself remarkably well to the requirements of crop improvement: the rapid assessment with high throughput of measurements which produce data-rich results. In particular, we expect that the development of monitoring and remote fluorescence techniques should play a key part in the development of automated crop phenotyping techniques; so far this has yet to be seen.

### *Common protocols for fluorescence analysis*

These are included to provide the user with some guidance for good practice and help to overcome some pitfalls in common situations. See Table 1 for a list of formulae. Usually devices will make calculations automatically, and the diligent user should compare these with the procedure below and refer to Fig. 2.

#### *A. Measurement of $F_v/F_m$*

(i) Dark-adapt leaf material for a minimum of 20 min. This can be achieved using the manufacturer's leaf clips or user-protocol (e.g. Pask *et al.*, 2012). The leaf must be kept darkened continuously for the entire process of measuring $F_v/F_m$.
(ii) Apply the fluorescence detector with the measuring beam off and ensure the reading is zero.
(iii) Switch on the measuring beam. Check for quenching induced by the measuring beam and adjust the measuring beam intensity accordingly. Usually this is only necessary once for a given set of plants/plant material.
(iv) Measure $F_o$
(v) Apply saturating pulse, typically 0.8 s at an intensity of at least 4000 µmol $m^{-2}$ $s^{-1}$. Attain $F_m$ value.
(vi) If possible check there is no quenching during the pulse.
(vii) Good practice: use non-stressed plant material to check $F_v/F_m$ of 0.81–0.83.

#### *B. To measure $F_q'/F_m'$ (ϕPSII) and NPQ at a known light intensity*

(i) If following on from (A) above, apply the actinic light immediately after measurement of $F_v/Fm$.
(ii) Good practice: ensure the material is at steady state. If using ambient light, ensure that the light intensity at the leaf surface is known and that it is stable during the measurement, in order that results have proper context.
From the dark-adapted state, this will usually take a minimum of several minutes and should be monitored using the $F'$.
From a non-dark adapted state using ambient light, it is advisable to wait until the $F'$ signal is stable.
(iii) Apply saturating pulse.
(iv) For NPQ it is essential to start with dark-adapted material (a measurement of $F_o'$ is not required). Following the steps in (A) and (B) above, NPQ is calculated as in Table 1.

#### *C. To measure $F_q'/F_v'$ (qP)*

(i) As in (B), but a measurement or calculation of $F_o'$ is required.
(ii) Following the saturating pulse, the actinic light is switched off and $F_o'$ measured after a few seconds. Accuracy is improved with the use of an FR light source to oxidize PSI, $Q_A$, and electron transport intermediates.
(iii) An alternative method for $F_o'$ estimation is possible if the $F_o$ has been previously calculated (see text).

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