Variable Chlorophyll Fluorescence – Overview (2015)

There have been some recent important changes, contributing to the understanding of plant physiology, in the areas of chloroplast migration, acute photoinhibition, mechanisms that balance Photosystem II and Photosystem I photochemistry, the times required to reach steady state photosynthesis, the times required for reliable dark adaptation, and in plant stress adjustment mechanisms. Much of this new information is taken from Cazzaniga, Bassi (2013), Dall’Osta, Bassi (2014), Laisk, Dall’Osta (2014), and Stand, Kramer (2015).

The meaning of the rise of variable chlorophyll fluorescence has been studied by many sage researchers and it’s details and understanding continue to advance. This document is designed as an introduction to the most widely held views on the subject with brief mention of alternative views. Much of this review is based on Zhu X-G., Govindjee, Baker N.R., deSturler E., Ort D.R., Long S.P. (2005) & Zhu X-G., Wang Y., Ort D.R., & Long S.P. (2012)

New research:

- Laisk, Dall’Osta (2014) explores and measures the PSII/PSI ratio, and the balancing of PSII and PSI output of ATP and NADPH under state 1 conditions. Page- 3
- Peterson, Laisk (2014) Using as custom optical device to measure PSII & PSI minimum fluorescence, they confirm that PSII reaction centers are present in bundle sheath cells of C₄ plants and that related proteins accumulate. It is also likely that chlorophyll a and b production help balance PSII/PSI function and light capture. Page 4
- Strand, Kramer (2015) finds that the production of H₂O₂ drives cyclic electron transport from PSI for the production of more ATP in PSII. Furthermore, H₂O₂ is the signaling molecule in plant stress response. Page 3.
- Cazzaniga (2013), & Dall’Osta (2014) confirm that chloroplast migration, an intense light avoidance mechanism, replaces the intermediate chlorophyll fluorescence change that was thought to be due to state transitions and acute photoinhibition at high actinic light levels. Pages 16-17, 28-29
- Loriaux (2013) refines the process of F₅₉’ correction in Y(II) and ETR measurements at high actinic light levels. Pages 23-24
- Vredenberg (2015) offers an alternative view of the OJIP variable chlorophyll fluorescence rise, using a modulated fluorometer. With short saturation pulses, followed by periods without actinic light, he measures three different fluorescence decay rates and estimates the fraction of Q₉ non-reducing PSII reaction centers. Page 14
As stated earlier, the origin of the rise variable chlorophyll fluorescence is chlorophyll “a” in conjunction with photosystem II (Zhu 2005, Zhu 2012). The variable rise continues along with electron transport mechanisms until the Cytochrome b6f complex re-oxidizes PQH2 to PQ. Light energy entering photosystem II can be converted to chemical energy by photochemistry. It can also be re-emitted as chlorophyll fluorescence or it can be re-emitted as heat. These three processes are in competition, so that when photochemistry output is high, chlorophyll fluorescence and heat are lower. Conversely, if fluorescence is maximized, then the other two paths are minimized.

While photosystem I does emit chlorophyll fluorescence as well, it is at a much lower level and it is not variable. For that reason, chlorophyll fluorescence of photosystem II is of much greater interest (Schreiber 2004). This minimum non-variable PSI fluorescence is part of minimum fluorescence $F_0$ values, part of maximum fluorescence $F_M$, part of steady state fluorescence in light adapted measurements, $F_s$, and part of chlorophyll fluorescence is light that is re-emitted at a longer wavelength after being absorbed by chlorophyll molecules at a shorter wavelength. Variable chlorophyll fluorescence is only observed in conjunction with chlorophyll “a” in photosystem II and the rise in fluorescence continues through electron transport until the re-oxidation of PQH2 (Plastoquinol) to PQ (plastoquinone) by the Cytochrome b6f complex (Zhu 2005). Maximum variable fluorescence, or $F_M$, occurs when QAQB$^-$ and PQH2 are at a maximum (Zhu 2005). By measuring the intensity and nature of variable chlorophyll fluorescence, using protocols that have been developed, plant physiology can be investigated (Baker 2004). The variable nature of chlorophyll fluorescence allows research into the light reaction of plants, plant photo-protection mechanisms, heat dissipation, chloroplast migration (Cazzaniga 2013 Dall’Osta 2014), state transitions (Laisk 2014), energy balance between photosystem II and photosystem I (Strand Kramer 2015), correlation with photosynthesis carbon assimilation, and measurement of most types of plant stress at usable levels (Baker 2004).

In C3 plants, about 30% of $F_0$ fluorescence is due to PSI, and in C4 plants about 50% of $F_0$ fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in $F_M$, in C3 plants, and about 12% of $F_M$ in C4 plants (Pfundle 1998). This not a problem when comparing $F_v/F_M$ measurements for plant stress because PSI fluorescence does not change. It remains constant.

Photosynthesis is comprised of a light reaction and a dark reaction. The light reaction converts light energy into chemical energy (ATP & NADPH) that can be used in the dark reaction (Calvin–Benson–Bassham cycle). The dark reaction uses the energy molecules NADPH and ATP, created by the light reaction, to produce simple sugars in conjunction with the assimilation of CO2 from the air. The relationship between the two is related, and may be correlated, but it is not always straightforward (Roosequist and van Kooten 2006). Correlation of variable chlorophyll fluorescence in the light reaction and carbon assimilation in the dark reaction has a linear relationship in C4 plants, and a curve-linear relationship in C3 plants (Schrieber 2004). This correlation in C3 plants can break down under special conditions that create photorespiration such as drought and heat stress. Under photorespiratory conditions, oxygen and possibly other electron sinks, alternatively use the energy molecules generated in the light reaction, even after leaf carbon assimilation has been reduced. Under these conditions, chlorophyll fluorescence levels may be unchanged until more severe plant stress occurs (Baker 2008), (Flexas 1999). In C4 plants, there is no significant photorespiration. Fortunately, special chlorophyll fluorescence assays or methods have been developed to overcome the most interesting problem areas including drought stress in C3 plants, nitrogen stress in C3 plants and C4 plants, and heat stress in C3 plants (Desk Top Plant Stress Guide).

Linear electron transport through PSII and PSI is light driven and allows extraction of electrons and protons from water and allows their transfer to the lumen on the inner side of thylakoid membranes. It is also
involved in translocation of protons from the stroma to the thylakoid lumen (Strand, Kramer 2015). The increased protons create an increase in pH with a proton motive force that pumps protons through the thylakoid membrane into the stroma surrounding the outside of the membranes. The electrons are transferred to plastocyanin in the lumen and eventually PSI. ATP is created in the stroma with help from the proton motive force from PSII. NADPH is also created in the stroma from continued electron flow through PSI and protons pumped through the thylakoid membrane due the ΔpH change in the thylakoid lumen (Strand, Kramer 2015). The proton motive force also regulates other metabolic functions in the chloroplast including the photoprotective xanthophyll cycle or qE, and down regulates electron transfer during linear electron transport by controlling the oxidation of PQH2 into PQ by the cytochrome b6f complex (Strand, Kramer 2015). Another source of electrons is cyclic electron transport. It occurs from PSI and it is used to increase ATP production that helps balance the ATP/NADPH ratio (Strand, Kramer 2015) (More information is given under the new information section listed below and the diagrams on page 9).

Three protons are released into the thylakoid lumen for each electron transported in PSII and PSI. ATP is thought to require 4.67 protons sent through the ATPase proton pump to achieve an ATP / NADPH ratio of 1.33 (Strand, Kramer 2015). This number is considered far to low to fill the needs for simple sugar creation in the dark reaction, and accomplish other ATP driven functions such as tRNA control of protein synthesis or translation, protein transport, or other functions. The ratio of ATP to NADPH creation changes with plant stress conditions, growing conditions, and possibly other factors (Strand, Kramer 2015).

New information about antenna systems, pigments, and energy balancing between PSII and PSI

It is important that the amount of ATP energy molecules created by PSII and NADPH energy molecules created by PSI reach an equilibrium, in proportion to the amounts required by the dark reaction (Calvin–Benson–Bassham cycle). If not, then toxins are produced and the plant will eventually be destroyed (Laisk 2015). It was thought that the “excitation partitioning between the photosystems is proportional to the ratio of the densities of photosystems. The latter generally indicates the dominance of PSII, varying from 2.5/1 to 1.2/1 depending growth light quality in pea”, a C3 plant (Melis 1996) (Laisk 2015). More recently, it was found that in Sun flower, a C3 plant, grown without light stress (State 1 conditions), at lower visible light levels, and including far red light (to drive PSI), there was a more complex answer. Laisk found that when measuring just the photosystem monomer, without the associated antenna complexes, the ratio of absorbed light partitioning of PSII to PSI reaction centers was about 1.01/1. However, light absorption by PSII, with its larger antenna complexes, compared to PSI and its smaller antenna complexes, was about 1.3/1 in favor of PSII. In addition, PSI showed a higher efficiency yield (electrons transported per photon absorbed) of 0.88 while PSII efficiency was lower at 0.63 per photon absorbed (Laisk 2014). Much of the difference in efficiency was found in electron losses that occur from excitation decay before charge separation, and recombination at QB in PSII, after charge separation (Laisk 2014). (Charge separation is the energy threshold that drives the light reaction of photosynthesis and starts the rise of variable chlorophyll fluorescence. All other reactions in PSII are down hill, from an energy point of view, in a series of oxidation-reduction reactions).

Other mechanisms are also involved in balancing ATP and NADPH output. The xanthophyll cycle, under high light or saturating light, partially shades PSII in the blue part of the spectrum and can reduce light absorption of the blue spectrum up to about 30%. PSII is more sensitive to intense blue light photoinhibition (Laisk 2014). Balancing of the photosystem outputs can also occur at high light levels due to photoinhibition, that reduces the number viable or QB’ reducing PSII reaction centers compared to PSI (Fan 2007), (Laisk 2014), (Zhu 2005). State transitions and cyclic electron transport from PSI also contribute to balancing of ATP and NADPH output by the two photosystems.
At low actinic light levels, state transitions can contribute up to about 67% of non-photochemical quenching (Lichtenthaler 1999) in state 1 conditions, and its affects are reduced as actinic light intensity increases. In the past, it was thought to contribute to about 10% of nonphotochemical quenching at high light levels (2000 μmol), but at this time, it is likely a non-factor at high light levels (Cazzaniga 2013) (Dall’Osta 2014). Instead, chloroplast migration, a light avoidance mechanism, has been shown to be responsible for, not only the effects previously thought to related to state transitions at high light levels, but it also the affects previously thought to be due to acute photoinhibition. Chloroplast migration reduces total leaf light absorption by increasing light transmission through the leaf and it has been shown to be responsible for up to 30% of NPQ at high light levels (Cazzaniga 2013) (Dall’Osta 2014). With this new research, it appears that the ratio of leaf absorbance or PSII/PSI varies with species, plant type (C3 or C4), light quality during growing conditions, and recent light history due to photoinhibition and state transitions (See the section on chloroplast migration and state transitions for more information on this subject, later in this document) (Laisk 1996), (Fan 2007) (Laisk 2014).

While the various mechanisms listed above primarily affect the linear electron transport found in both photosystem II and photosystem I, there is also a cyclic electron transport mechanism that occurs from Photosystem I, providing electrons to the chloroplast stroma, in an attempt to balance the chemistry of the two photosystems and produce more ATP. Cyclic electron transport has been known for some time. However, Strand, Kramer (2015) recently found that cyclic electron transport was driven by the creation of hydrogen peroxide (H2O2). When there is a deficit of ATP necessary for creation of simple sugars and other chloroplast functions, more H2O2 is produced under photorespiratory plant stress conditions. H2O2 is likely the missing link to plant stress response (Strand, Kramer 2015).

In a work by Peterson & Laisk (2014), using a specially constructed optical device, to measure chlorophyll fluorescence emission of PSII at 680 nm and PSI at 750 nm, they were able to determine the ratio of PSII to PSI reaction centers. The maize sample was illuminated at F0, low light levels (state 1), using a 595 nm amber LED. Using Laisk 2014 research in conjunction with their current research, they were able to determine that PSII reaction centers were present in maize bundle sheet cells. Furthermore, they found evidence of another non-variable chlorophyll fluorescence source, in young leaf tissue, that diminished with Chlorophyll b development as the leaf matured. As a result, they believe that in young tissue, there is a surplus of PSII proteins, a lack of bound chlorophyll, and “the balance in PSII-PSI function and light capture is regulated by coordinated synthesis of Chlorophylls a and b”.

**How does the variable chlorophyll fluorescence work?**

Upon illumination of a leaf that is dark-adapted overnight, or dark adapted by artificial means, there is a rapid rise in fluorescence from Photosystem II (PSII), followed by a slow decline. This displays the variable nature of chlorophyll fluorescence in photosystem II. It was first detailed by Kautsky in 1931, and this is called the Kautsky Effect. (Govindgee 2004) (Kautsky 1931)

In Photosystem II, light is absorbed by accessory pigment-protein complex molecules called antenna (Zhu 2005), and transferred to PSII reaction centers. Zhu divides antenna into peripheral antenna and core antenna. Core antennae are tightly bound chemically and adjacent to the reaction center. Peripheral antennae are near the core and can be chemically bound strongly, at a medium level, or loosely. They are; however, in close proximity to either photosystem II or photosystem I reaction centers (Schneider 2013). In the photosystem II reaction center are D1 and D2 pigment-protein complexes that coordinate the specialized chlorophyll “a” photoactive reaction center structure, P680. (Papageorgiou 2004). There are different models that show P680 acting as various types of dimer (Papageorgiou 2004), (van Gronelle 2004), (Razewski 2008), or a monomer (Takehashi 2009); however, the Zhu (2005, 2012) papers, written by some of the most prominent chlorophyll fluorescence researchers, focus on fluorescence and provide a conservative approach that is currently the most accepted. The core antenna complexes are known as CP43
and CP47. They are chlorophyll-protein complexes that are adjacent and associated with the D1D2 P680 PSII reaction center. (For more details concerning these structures see Diagrams C & D).

There are several different pigment types associated with peripheral antenna including: Chlorophyll a, Chlorophyll b, lutein, xanthophylls, beta carotene, and lycopene. The antennae absorb light in different wavelength ranges, and transfer the energy to nearby photosystem I and II reaction centers. As the energy transfer occurs to the reaction center, a small amount of energy is lost to passive heat loss. The antennas transfer energy to photosystem II reaction centers and to photosystem I reaction centers. Both photosystems are located in thylakoid membranes. Thylakoids are lumened structures stacked, inside plant chloroplasts. They are pictured in the drill down diagrams below (Diagram B). There are two basic types of photosystems called either photosystem II, or photosystem I. (Diagram C). While it has been shown that chlorophyll “b” can show a slight fluorescence when energy can not be transferred to chlorophyll “a”, the emission spectrum in the 660 nm to 665nm range, is normally filtered out by chlorophyll fluorometers and it does not directly affect F_O, or F_V/F_M (Govingee 1978). There has been no fluorescence observed in chlorophyll “b” when energy transfer to chlorophyll “a” is normal. (Govingee 1978).

There are also two varieties of photosystem II reaction centers that affect variable chlorophyll fluorescence. Energy is transferred to either Q_B reducing reaction centers, that are capable of being used in photochemistry, or to Q_B non-reducing reaction centers, that are not capable of photochemistry. Q_B non-reducing reaction centers do not transfer their energy to other reaction centers, and the absorbed energy is reemitted as either heat or non-variable chlorophyll fluorescence at a low level. Q_B non-reducing reaction centers have smaller core antenna, an oxygen evolving complex, and no peripheral antenna. There is also no electron transfer beyond Quinone A or QA. (Quinone B is designated Q_B). A higher number of Q_B non-reducing reaction centers in the leaf therefore increase the minimum fluorescence, F_O, which is measured in a dark adapted state, and decreases the F_V/F_M measurement parameter to be discussed in detail later (Zhu 2005).

Q_B reducing reaction centers that can be used in photochemistry, can be either opened or closed. They are open if they are chemically oxidized, and they are closed if they have been chemically reduced. Closed Q_B reducing reaction centers can transfer additional energy to other open Q_B reducing reaction centers. In a properly dark adapted state, most or all Q_B reducing reaction centers will be open (Zhu 2005).

When a, Photosystem II, Q_B reducing reaction centers receive an adequate threshold of light energy, it drives something called charge separation that occurs in photosystem II (Zhu 2005).

This, and the electron transfer to the AO molecule in photosystem I are the only steps where light energy is converted into chemical energy (Zhu 2005).

At charge separation in photosystem II, an electron is transferred from P_680, the primary electron donor, to the primary electron acceptor, pheophytin. The chemical process for charge separation is shown in diagram A. During this process, an electron is added from Tyrozine, Y_Z, generated from the Oxygen Evolving Complex, through the Mehler or water reaction. The oxygen evolving complex involved in this process is also shown in the diagram C below (Zhu 2005).

The energy levels of the remaining steps in the light reaction of photosystem II are all down hill in oxidation-reduction reactions (Zhu 2005).

Measuring fluorescence in a dark adapted state, starts by measuring minimum fluorescence generated by peripheral and core antenna before any Q_A has been chemically reduced (Zhu 2005). This is commonly done using a modulated chlorophyll fluorometer that excites minimum fluorescence. The modulated light source is adjusted high enough to allow minimum antenna fluorescence measurement, but it is set low enough to
prevent the reduction of any QA. Additional sources of minimum fluorescence also include Photosystem I, and PSII QB non-reducing reaction centers (Zhu 2005), (Opti-Sciences Fv/Fm checklist application note).

The rise of variable chlorophyll fluorescence starts at charge separation and continues through the reduction of QA, QB, and the reduction of the Plastoquinone Pool. Evidence shows that the rise ends with the re-oxidation of PQH\textsubscript{2} (Plastoquinol) to PQ (plastoquinone) by the Cytochrome b\textsubscript{6}f complex. Maximum variable fluorescence or F\textsubscript{M}, occurs when QAQB\textsuperscript{2-} and PQH\textsubscript{2} are at a maximum (Zhu 2005). The height of F\textsubscript{M} is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH\textsubscript{2} to PQ. A higher, k\textsubscript{ox}, reoxidation constant, or a larger plastoquinone pool reduces the F\textsubscript{M} value (Zhu 2005).

![Diagram A](image)

Measuring screens from the OS30p+ and the OS1p

F\textsubscript{O} represents minimum chlorophyll fluorescence before any QA has been reduced.

F\textsubscript{M} is maximum variable chlorophyll fluorescence when QAQB\textsuperscript{2-} and PQH\textsubscript{2} are at a maximum.

F\textsubscript{O} is affected by the number of the number of QB non-reducing reaction centers. Higher numbers raise F\textsubscript{O}. It is also affected by the ratio of PSII to PSI reaction centers. In C\textsubscript{3} plants, where the ratio of PSI reaction centers is higher than in C\textsubscript{3} plants, PSI non-variable fluorescence can represent up to 50% of F\textsubscript{O} (Schreiber 2004). It is also affected by dark adaptation status and setting the modulated light correctly. PSI gives off a low non-variable chlorophyll fluorescence.

The height of F\textsubscript{M} is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH\textsubscript{2} to PQ. A higher, k\textsubscript{ox}, reoxidation constant, or a larger plastoquinone pool reduces the F\textsubscript{M} value (Zhu 2005). It is also affected by using a proper dark adapted state to relax the xanthophyll cycle, \( \Delta P \)H of the thylakoid lumen, state transitions or chloroplast migration (Cazzaniga 2013). In addition it can also be affected by light history as it applies to acute photoinhibition and chronic photoinhibition (Lichtenthaller 2004). Chloroplast migration caused by high light levels, relaxes in 35 minute or less, while chronic photoinhibition, caused by several hours of high light levels, starts to relax or repair after 40 minutes, and may take from 40 to 60 hours to relax or repair (Lichtenthaller 2004). This means that there will likely be some photoinhibition that is built into field plant measurements after a sunny day. For this reason, it is important to compare samples with a similar light history, or take this variable into account when designing experiments. (See the Opti-Sciences quenching application note for more details. See the Opti-Sciences Desk Top Plant Stress Guide for using F\textsubscript{V}/F\textsubscript{M} along with recommendations and references regarding specific types of plant stress.)

Note: Many research reviewers like “overnight dark adaptation” even for F\textsubscript{V}/F\textsubscript{M}. It is recommended that you check with your target publications to comply with their views before experimental design.
Photochemical and chemical reactions involved with variable chlorophyll fluorescence of PSII in a $Q_B$ reducing reaction center.

Fo is minimum chlorophyll fluorescence that is measured from antenna after dark adaptation and before any $Q_A$ has been chemically reduced.

$F_M$ is maximum variable chlorophyll fluorescence that occurs when $Q_A, Q_B^+$, and PQH$_2$ are at a maximum. The height of $F_M$ is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of Plastoquinol to Plastoquinone.

The fluorescence rise ends with the reoxidation of PQH$_2$ by the cytochrome $b_{5f}$ complex.
Drill down diagram of variable and non-variable chlorophyll fluorescence

(The diagram shows a detailed comparison between C3 and C4 plant leaves, focusing on structural and functional differences. It includes close-up views of chloroplasts and stroma lamellae, highlighting the arrangement and distribution of chlorophyll in these different plant types.)

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PSII rich membrane - PSII supercomplex formations, found in stacked thylakoid membrane areas, can be found in a disorganized liquid form, an organized crystal form, or in intermediate forms.

In state transitions, LHCII antenna become phosphorylated, disassociate, and migrate to stroma lamella or marginal regions where they attach to PSI reaction centers. The mechanisms are under debate.

Shape models and descriptions were adapted from:
Three types of reaction centers

Reactions from one $Q_B^-$ reducing PSII reaction center
The portion of the light reaction involved in the rise of Photosystem II variable fluorescence.

Photosystem II

$F_O$, the minimal fluorescence, is dark adapted antenna fluorescence before any $Q_A$ has been thermally reduced.

$F_M$, the maximal fluorescence, occurs when $Q_A^+$ and $Q_B^-$ are at a maximum & the rise in variable fluorescence ends with the reduction of $Q_B^-$ by the cytochrome bc$_{1}$ complex.

Stroma

Fluorescence Heat ↑

Thylakoid Lumen

Represents electron transfer
Represents proton transfer

Oxygen Evolving Complex: OEC

PSI reaction center

Non-variable fluorescence of PSI contributes to $F_O$ and $F_M$. In C_4 plants, about 30% of $F_M$ fluorescence is due to PSI, and in C_3 plants about 50% of $F_M$ fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in F_M in C_4 plants, and about 12% of $F_M$ in C_3 plants.

$e^-$ from Ferredoxin in PSI goes to the Cytochrome bc$_{1}$ complex in a process called cyclic electron transfer.

Stroma

Fluorescence Heat ↑

Thylakoid Lumen

Represents electron transfer
Represents proton transfer

$P_{700}$, the Photosystem I reaction center.

Low, non-variable fluorescence
Photosystem II $Q_B^-$ non-reducing reaction center not used in photochemistry.

PSI $Q_B^-$ non-reducing reaction centers are not used in photochemistry.

Increased numbers of these reaction centers increase minimum fluorescence, $F_O$, and therefore decrease $F_M$.

Stroma

Fluorescence Heat ↑

Thylakoid Membrane

$Q_B^-$ non-reducing PSII reaction centers have smaller core antenna, no OEC, no peripheral antenna.
D₁ and D₂ are a pigment-protein complex that coordinates the photoactive reaction center P680, a specialized chlorophyll "a" structure that includes two chlorophyll "a" molecules. Different PSII models show P680 acting as various types of dimer, or acting as a monomer. This is where charge separation occurs. (Papageorgiou 2004) (van Grondelle 2004) (Raszewski 2008) (Takehashi 2009)

CP₄₃ and CP₄⁷ are chlorophyll-protein core antenna complexes that are adjacent and associated with the D₁D₂ P680 PSII reaction center. (van Grondelle 2004)

X-ray diffraction shows that the PSII core that includes the D₁D₂ P680 PSII reaction center, as well as the CP₄₃ and CP₄⁷ chlorophyll-protein core antenna complexes, contain more than thirty chlorophyll "a", two pheophytins, from two to seven carotenoids, two plastoquinones, two bicarbonates, one nonheme iron, an oxygen evolving complex, and intermembrane α-helices. (Papageorgiou 2004) (van Grondelle 2004)

LHClI, or light harvesting complex for PSII, are peripheral antenna that include three types of proteins, seven chlorophyll "a" molecules, five chlorophyll "b" molecules, and a variable amount of violaxanthin and zeaxanthin. In dark adapted leaves, there can also be an extra lutein, and a neoxanthin. (van Grondelle 2004)

CP₂₄, CP₂₆, and CP₂⁹ are chlorophyll-protein peripheral antenna that are minor versions of the larger LHClI peripheral antenna. They contain between eight and ten chlorophylls of both a and b types. They also contain several xanthophylls. While they only contain about 15% of the chlorophylls in PSII, their proximity to the core may be important in regulatory mechanisms. (van Grondelle 2004)
OJIP and F_V/F_M - Understanding the fluorescence rise and steps.

If the rise in chlorophyll fluorescence is examined with high speed time resolution in the range of microseconds, and milliseconds, specific steps appear during the rise. The following descriptions represent a synopsis of information available from a paper written by Xin-Guang Zhu, Govindjee, Neil R. Baker, Eric deSturler, Donald R. Ort, and Stephen P. Long in 2005. The information was reaffirmed in a second paper in 2012 by Xin-Guang Zhu, Yu Wang, Donald R. Ort, and Stephen P. Long. These are some of most respected names in chlorophyll fluorescence and photosynthesis. While there is still some debate regarding some of the details of the OJIP fluorescence rise, this approach is the one that is most accepted. For an alternative interpretation see Vredenberg 2015. Vredenberg is also a highly regarded researcher.

O – is commonly measured at 20 µsec. after the start of actinic illumination in continuous fluorometers, and is not equal to F_O measured by modulated fluorometers. Continuous fluorometers use linear regression analysis to estimate F_O, or minimum fluorescence in a dark adapted state, before any QA has been chemically reduced.

The height of both O and F_O are affected by the ratio of the size of peripheral antenna to core antenna. A larger ratio of peripheral antenna causes O and F_O to be lower.

Both O and F_O are affected by the ratio of Q_B^- non-reducing reaction centers to Q_B^- reducing reaction centers. As the ratio of Q_B^- non-reducing reaction centers increases, both O and F_O values increase. This is shown in the bottom diagram. The green line represents a sample with significantly fewer Q_B^- non-reducing reaction centers, and the red line represents a much higher percentage of Q_B^- non-reducing reaction centers.

O to J – The slope of the rise in fluorescence is affected by the probability that excitation energy will migrate from a closed Q_B^- reducing reaction center to an open Q_B^- reducing reaction center. Higher probability delays the rise as shown in the lower diagram in dark blue. Q_B^- non-reducing reaction centers do not transfer energy to open Q_B^- reducing reaction centers. Energy absorbed by these reaction centers is converted to heat and fluorescence only. They are not involved in photochemistry. The O-J rise represents the photochemical reduction of pheophytin and QA. J represents maximum values for QAQ_B^- and QA'Q_B^- . J becomes more defined and lower if the dark adapted Oxygen Evolving Complex ratio of the specific states of S_1 to S_0 move from 1:0 to 0:1. The Dip after J becomes more defined with a higher S_0 value. It provides a greater P_{680}^+ concentration that is a strong fluorescence quencher. This dip is shown in the lower diagram in light blue. A separate new step called the K step can appear at 300 µsec. It only appears at high light levels (Vredenberg 2004), when there is severe nitrogen, iron, or sulfur deficiency (Strasser 2004). The Zhu 2005 paper show that the timing of J is somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 2 msec.
J to I - This rise represents the photochemical reduction of $Q_B^-$. “I” represents the first shoulder in the $QAQ_B^2$ chemical equation that ends at $P$ with a maximum for $QAQ_B^2$-$$. If properly dark adapted, the J to I rise starts with the ratio of $Q_B^- : Q_B^2-$ = 1:0 and ends with the ratio at 0:1. The dark adapted ratio prior to light exposure of $Q_B^- : Q_B^2-$ affects the slope and height of “I” as shown in the lower diagram by the yellow line.

Again, the Zhu group shows that the time to reach I is also somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 30 msec.

$$P = F_M$$

or Maximum variable chlorophyll fluorescence. This value represents a maximum for chemical values of $QAQ_B^2$, & $PQH_2$. The rise in fluorescence ends with the cytochrome $b_6f$ complex re-oxidizing $PQH_2$ to $PQ$. The height and slope of the rise to $P$ or $F_M$ are affected by the reoxidation rate constant of $PQ$, $k_{ox}$, and by the size of the plastoquinone pool. A higher rate constant and a larger PQ pool reduce the value for $P$. The time to reach $P$ is variable in the Zhu paper and in the Strasser JIP protocol. A high rate constant reduces the time to reach $P$, and a larger PQ pool extends the time to reach $P$. The time to reach $P$ is reported in the Strasser protocol. A larger PQ pool is shown in the lower diagram in dark red crimson.

In 2004, Wim Vredenberg discovered that the OJIP graph changes dramatically at different actinic light levels. In fact, the K step only appears under very high light levels, and under specific severe plant stress conditions. (Vredenberg 2004) For this reason, it is common to calibrate the light source of OJIP instruments, to ensure comparable results. The OS30p+ provides automatic actinic light calibration when the instrument is turned on.

The quality of light can also be a factor. It has been found that red actinic light penetrates the entire leaf, while blue light does not. For this reason, it has been common to compare work done using the same type of saturating actinic light sources. The OS30p+ offers a red calibrated light source with intensities that may be set at 3,000 μmols as found in Strasser’s earlier works, or 3,500 μmols in later works for the Strasser protocol, and up to 6,000 μmols for other work. Various light sources are used for measurement of $F_v/F_M$. Industry options include, red, red and blue, and white light halogen, LED, halogen and Xenon light sources. A paper comparing xenon and red light saturating light sources provided results that correlated well with slightly lower values using a red light source (Cessna 2010). The paper found poorer correlation with blue saturating light. For longer light adapted measuring protocols, used in quenching measurements, and light curves, or for extended pre-illumination of shorter light adapted tests, white actinic light sources have advantages, or at least illumination with equal amounts of red and blue actinic light. The apertures of plant stoma are mediated by blue actinic light (Kinoshita 2001). Chloroplast migration, as it occurs in nature, is responsible for up to 30 % of NPQ at high light levels, and only occurs under intense blue light or intense white light, not intense red light (Cazzaniga 2013). In addition, the absorption ratio of red to blue light changes with light intensity (Bernacchi 2002). Other light quality factors affect light adapted chlorophyll fluorescence measurements as well. They will be discussed later.
An alternative view:

The cause and meaning to the OJIP chlorophyll fluorescence rise, has been subject to more than one view for some time. Dr. Wim Vredenberg has been a champion of the “Three-state trapping model”. Unlike the details of the rise shown above, he shows that “Full reduction of QA is neither necessary nor required to reach \( F_M \)” (maximum dark adapted chlorophyll fluorescence).

Vredenberg uses short saturation pulses, followed by periods without actinic light to drive photosynthesis. Using a modulated light, he measures the chlorophyll fluorescence effects during the light and the dark periods, during different phases of the OJIP rise, including: O-J, J-I, and I-P. He finds three different rates of fluorescence decay, measured during the dark periods, contribute to the understanding of quenching, of the fluorescence rise, chemistry, and the physics of the OJIP rise.

In his model, it takes the PSII electron acceptor pair, Pheophytin and QA (or Quinone A), to act as a two electron trap pair, that requires two single turn over light flashes to semi-close and then completely close (chemically reduce) Pheophytin and QA.

He finds that O-J is the result of QA reduction. J-I is a phase where more than 50% of QA is reduced, there is re-quenching of QA, there is an increase in the slowest version decay of the fluorescence signal in the dark, and there is a pH change at the QA – QB site. The I-P phase results from a build up of the proton motive force in the thylakoid lumen and cyclic electron transport from PSI.

Vredenberg has developed methods for measuring the portion of QB- non-reducing reaction centers, and developed the FIAFLU algorithm (fluorescence induction algorithm) that provides a strong prediction of the actual OJIP rise from readings taken. The intensity of slowest rate of decay, at 0.25 ms in the dark, after a short saturation flash, provides an estimate of the fractional size of QB- non-reducing PSII reaction centers. (The Vredenberg protocol is currently available on the Opti-Sciences OS5p+ chlorophyll fluorometer)

**Photochemical and Non-photochemical quenching.**

After proper dark adaptation to a known state, a leaf is exposed to a photosystem saturating light. Initially, a maximum amount of the saturating light, absorbed by the leaf, and used in \( F_v/F_m \), goes to variable chlorophyll fluorescence with smaller amounts going to unregulated heat dissipation and photochemistry. The same thing is true when using a saturating actinic light in OJIP protocols for the initial rise of fluorescence.

There are mechanisms, that are slower reacting, that also affect variable chlorophyll fluorescence. After dark adaptation, and the initial rise in chlorophyll fluorescence, these mechanisms begin to respond. Depending on the type of plant, peak fluorescence is maintained from 0.5 seconds to 1.5 seconds in land plants (Schreiber 1995), and from 25 milliseconds, to 50 milliseconds in algae (Schreiber 1995). The fluorescence output then begins to drop due to the initiation of photosynthesis where more light is used in photochemistry, a process called photochemical quenching. This, and photo-protective mechanisms, start to adapt to existing actinic light levels. The xanthophyll cycle and the \( \Delta \) Ph of the thylakoid lumen convert absorbed light into regulated heat dissipation, a form of non-photochemical quenching, considered to work as photoprotective mechanisms. At higher actinic light levels, the xanthophyll cycle screens out about 30% of blue light absorbed but not red light (Laisk 2014). At higher light levels, there is more non-photochemical quenching. This process takes about several seconds to minutes in greenhouse plants (Lichtenthaler 2004),

(new information)
but it can take up to seven minutes in field plants (Baker 2008). qE is a parameter that is used to measure the non-photochemical quenching of the photoprotective xanthophyll cycle and ΔpH of the thylakoid lumen mechanisms. This parameter is used in conjunction with quenching relaxation protocols (Muller 2001). qL from the Kramer lake model and qP from the puddle model are parameters designed to measure photochemical quenching or a measure of open PSII reaction centers.

There are still other slower acting mechanisms that continue to lower fluorescence output after the initial fluorescence rise. Since a balance of ATP produced by PSII and NADPH produced by PSI production, in the light reaction, is required for optimal photosynthesis, it is assumed that the global yield of PSII and PSI must be proportional. While sometimes it is not (Laisk 2014), there are several mechanisms involved in trying to balancing the output. Therefore, understanding how the two systems come into balance has been of interest. Some prominent researchers see evidence that these intermediate but slower component mechanisms, that are part of an NPQ (or non-photochemical quenching) measurement, may not be the same in all photosynthetic organisms and at different light levels (Cazzaniga 2013) (Dall’Osta 2014). There is likely a relationship between the phosphorylation found in state transitions and NPQ regulation found in some monocots (corn, barley and rice) at lower light levels. There is also some strong evidence that qT fluorescence, or fluorescence from state transitions, exists in the green algae Chlamydomonas reinhardtii at lower light levels. (Depège N., Bellafiore S., Rochaix J-D., 2003). At high light levels and near saturating light levels, photoinhibition affects the photosystem balance by disabling the number of PSII reaction centers (Laisk 2014). However, there are times when PSII and PSI do not achieve balance and one or the other photosystem is over stimulated for optimal photosynthesis (Laisk 2014).

Another mechanism that acts as a photoprotective mechanism, through light avoidance, is chloroplast migration. It can be responsible for up to 30% of NPQ at near saturation light conditions. (Cazzaniga 2013) (Dall’Osta 2014). The relevance of state transitions measured as qT fluorescence is likely valid for low light levels but not at high light levels (Cazzaniga 2013), (Dall’Osta 2014). The evidence shows that changes previously reported as qT in quenching relaxation tests are not due to state transitions at higher light levels or saturating actinic light intensities. The latest evidence points to chloroplast migration and the resulting reduced leaf photon absorption as the source of intermediate fluorescence change during light adaptation, during dark adaptation, and during quenching relaxation, in dicot land plants at the very least (Cazzaniga 2013). Furthermore, the evidence shows that what was thought to be acute photoinhibition, a change that occurs for up to a 30 minute time scale, is actually chloroplast migration. (Cazzaniga 2013) (Dall’Osta 2014).

The understanding of what happens during this intermediate time scale NPQ fluorescence change that is greater than a few minutes and less than thirty five minutes has changed recently. In 2010 (Nikens) it was found that qT did not exist at near saturation light conditions. It was thought that it must be an unknown slower acting mechanism related to zeaxanthin, and it was named qZ (Nilkens 2010). Since that time, there has been new evidence. Under higher light levels and near saturating light conditions, the latest data supports chloroplast migration as the source of fluorescence change and quenching relaxation, instead of state transitions, qZ or acute photoinhibition. This research also affects the type of actinic light sources that should be used to measure most light adapted parameters, quenching measurements, and under high light conditions. It also could affect the times recommended for dark adaptation, and the time required to reach steady state photosynthesis under high light conditions. (Cazzaniga 2013), (Dall’Osta 2014).

Until recently, it was believed that as a plant goes from a dark adapted state to a light adapted state or from a light adapted state to a dark adapted state, there were three basic mechanisms involved in chlorophyll fluorescence measurement of nonphotochemical quenching (NPQ). Those mechanism parameters were qE, qT, and qI. qE can be described as a rapid photo-protective adjustment of photosystem II caused by ΔpH of the thylakoid lumen and the xanthophyll cycle. It can take qE several seconds to minutes to adjust, and it tends to be longer in field plants (Baker 2008, Murchie 2011, Nilkens 2010). Traditional qT was thought to
be caused by state transitions. $q_T$ could take up to fifteen or twenty minutes has been described as a fluorescence change that overlapped somewhat with $q_E$. Changes that took longer were related to $q_I$ or photoinhibition (Ruban 2009). There is now significant evidence to show that the fluorescence change measured as $q_T$ in quenching relaxation measurements is likely due to other chloroplast migration at least under high actinic light conditions in many land plants. (Cazzaniga 2013).

State transitions – classical view:

According to classical state transition theory, state transitions are thought to be a low light level survival mechanism that allows balancing of light between photosystem II (PSII) and photosystem I (PSI). It was believed that LHCII antenna trimers, or peripheral phosphorylated light harvesting complex II antenna, migrated from PSII complexes, to PSI complexes. The movement would occur from one thylakoid membrane to another when they were very close or adjacent to one another. The movement and reaction took place on the stroma side of the thylakoid membrane allowing the LHCII antenna to serve as a PSI antenna. When de-phosphorolated, the LHCII antenna favored movement back to PSII. The result was that at low light levels, movement was favored to PSI. LHCII phosphorylation was a prerequisite for dynamic regulation of relative balance of PSI/PSII excitation under artificially induced state transitions with different qualities of light. However, this process has not been viewed, without reservation, in PSII rich thylakoid membranes.

State transitions – a more recent view:

A more recent view of state transitions is as follows: There is no clear evidence to support the actual movement of LHCII (light harvesting complex II antenna) in stroma-exposed PSII rich thylakoid membranes, to PSI reaction centers. LHCII phosphorylation does not collect light energy for PSI in these cases. (Tikkanen 2012). Only in the margins of the grana thylakoid membrane do LHCII antennae behave according to the traditional view of phosphorylation-induced state transition (Tikkanen 2008). Tikkanen also states that there is substantial evidence to show that the classical mechanism of state transitions is not the sole method for energy balance between the two different photosystem types. There is also evidence to show that LHCII phosphorylation probably connects the regulation of light balance between PSII (photosystem II) and PSI (photosystem I) through unknown non-photochemical quenching mechanisms (Tikkanen 2012), that work not only with PSII but also PSI (Tikkanen 2010). It was also noticed that when light intensity is increased, the PsbS protein is protonated, turning the LHCII antenna into a dissipative state for PSII. (Li 2004, Tikkanen 2012). At lower light levels, LHCII activity is restored, and PSI activity is increased. Furthermore, phosphorylation is controlled by the enzymes STN7 and STN8 kinases and their opposing phosphatases, that are in turn, closely controlled by light intensity. These kinase functions are completely synchronized with PsbS and the xanthophyll cycle (Tikkanen 2012).

More recently, work done by Laisk (2014) shows that the balancing of PSII and PSI reaction centers is accomplished by a number of processes. It is accomplished through screening of blue light excitation of PSII by the xanthophyll cycle, and photoinhibition of PSII reaction centers at higher light levels. At lower light levels it has been found that either PSII or PSI can be over excited in relation to the other at different wavelengths of light and different ranges of wavelengths. Balance of PSII and PSI production is also accomplished by cyclic electron transport from PSI to enhance the creation of ATP in the chloroplast stoma, triggered by hydrogen peroxide production, H$_2$O$_2$ (Stand, Kramer 2015). Stand, Kramer (2015) found that the signaling molecule H$_2$O$_2$ was the likely the missing link in most plant stress response mechanisms.
**q_z - due to an unknown longer term xanthophyll cycle mechanism**

In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that under saturation light conditions, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were complete by 30 minutes. It was proposed that under steady state, saturating light conditions, NPQ should be divided into q_E, q_Z, and q_I.

As described by others, q_E is a process that is created and relaxes in the ten second to two hundred second time frame, and is depends on ΔpH of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from one hundred seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E is regulated the ΔpH of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the ten to thirty minute time frame. PsBs is not involved in q_Z, but is wholly dependant on zeaxanthin formation. Relaxation depends on the re-conversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they said that is was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was zeaxanthin devoid mutant npq1.

Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

**q_M – due to chloroplast migration**

Cazzaniga S, Dall’Osto L., Kong S-G., Wada M., Bassi R., (2013), (Dall’Osta 2014) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change, previously thought to be the result of state transitions and acute photoinhibition, or more recently, thought to be a longer lasting xanthophyll cycle process, was caused, by chloroplast migration. They found, as others have, that under high light levels, chloroplasts move from the top of cells to the sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research concludes that the cause of q_M is a decrease in light photon absorption which creates lower fluorescence yield, rather than a true quenching process. As mentioned earlier this is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity change mirrored the previously used q_T and acute photoinhibition. Time scales for chloroplast migration range from 20 minutes to 30 minutes in the wild plants tested, and up to 35 minute in some mutants. Chloroplast migration has been known and studied for a while, and it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga paper is the first to name chloroplast migration as the source of the q_T, q_Z and acute photoinhibition fluorescence change.

In addition, researchers found that high white actinic light and high blue actinic light allow chloroplast migration to occur as it does in nature. However, high red actinic light does not allow chloroplast migration to happen as in nature. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa T. 2001). Arabidopsis mutants that were devoid of q_E (npq4) were tested and Arabidopsis mutants devoid of q_E and chloroplast migration (npq4 photo2) were also tested along with other mutants. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutant that covered the remaining spectrum of mechanisms that affected chlorophyll
fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, acute photoinhibition, and state transitions as sources of $q_M$.

In regard to $q_T$ being replaced by $q_Z$, it was found that by using mutants devoid of $q_E$ and zeaxanthin, that the magnitude of $q_M$ did not change but the recovery time in the dark was longer. Plants were grown at 150 μmol photons m$^{-2}$ s$^{-1}$, and tested at 400 μmol photons m$^{-2}$ s$^{-1}$, 800 μmol photons m$^{-2}$ s$^{-1}$, and 1,200 μmol photons m$^{-2}$ s$^{-1}$. The adjustment time for $q_M$ ranged up to 30 minutes in wild plants and up to 35 minutes for some mutants.

The Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) observation that the stn7 mutant, devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of $q_T$ in Arabidopsis.

The fact that higher intensity white or high intensity blue actinic light is required to activate $q_M$, or chloroplast migration, indicates the need for either a white light source or a high intensity blue light and red light instead of using a high intensity red and lower intensity blue actinic light (Cazzaniga 2013). This new research shows that measuring artifacts are possible when using a low intensity blue light source for chlorophyll fluorescence measurement. ETR or $J$, Y(II) or $\Phi_{PSII}$, NPQ, $g_M$, $C_C$ and $q_I$ may all include measuring errors without a reliable actinic light source.

This also may change the times required for proper dark adaptation measurements, and the time to reach steady state photosynthesis under light adapted conditions. Until now, Maxwell K., Johnson G. N, (2000) has been the most sighted paper for reliable steady state photosynthesis conditions at any given light level. It lists 15 to 20 minutes as the time required for 20 wild land plants to reach steady state photosynthesis. Prominent researchers, Lichtenthaler (1999) and Ruban (2009), list the dark adaptation time required for quenching relaxation of $q_T$ at the same 15 to 20 minutes. With this new evidence from Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013), it shows that dark adaption times and the time to reach steady state lighting conditions should be extended, at least at higher light levels. They show that it takes from 20 minutes to 35 minutes for chloroplast migration to adapt to higher light levels and for relaxation in the dark. (Cazzaniga 2013)

Note: Many research reviewers like “overnight dark adaptation” even for $F_V/F_M$. It is recommended that you check with your target publications to comply with their views. Sometimes, tradition gets in the way of science.

**Photoinhibition** (new information)

Photoinhibition is a process that can occur at high light levels for extended periods of time. It was thought that the effects of high light levels for shorter periods of time, an hour or two, was a form of acute photoinhibition could be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter 1998). Recent research indicates that this likely due to chloroplast migration (Cazzaniga 2013).

Chronic photo-inhibition, caused by several hours of high light exposure, starts to relax or repair at about 40 minutes in the dark, and may take 30 to 60 hours to fully relax or repair under dark adaptation (Lichtenthaler H. & Babani F. 2004) (Theile, Krause & Winter 1998). Under low light history conditions, there are more PSII reaction centers in C₃ plants than PSI reaction centers (Laisk 2014). Photoinhibition targets PSII reaction centers (Laisk 2014) and at high light levels, it tends to balance the production of ATP by PSII reaction centers with the amount of NADPH produced by PSI reaction centers (Laisk 2014).
When making longer quenching and quenching relaxation parameter measurements related to photo-inhibition and photo-damage, it can take days for full relaxation or repair of the non-photochemical quenching parameter, $q_I$, or chronic photoinhibition, to pre-stress conditions. Photoinhibition is common in chronic high light stress, high heat stress, cold stress and over wintering stress. One should understand that most research reviewers require overnight dark adaption with either predawn measurement, or an artificial pre-dawn measurement for quenching measurements. However, to get a group that is not affected by photoinhibition, it may be necessary to subject samples to lower light levels for periods of up to 60 hours to ensure that photoinhibition has been eliminated. (Maxwell and Johnson 2000), (Lichtenthaler H. & Babani F. (2004). It is expected and accepted that there is some residual unrelaxed photoinhibition or NPQ in field plants using dark adapted samples, after sunny days, in the summer time. For this reason, it is important to compare samples with similar light history (Light history application note) Never compare any of the non-photochemical quenching values in samples that do not have the same FV/FM because FV/FM is the yard or meter stick used to determine these values. For more information on quenching measurements, refer to the OSI application note on quenching measurement.

Note: Many research reviewers like “overnight dark adaptation” even for FV/FM. It is recommended that you check with your target publications to comply with their views. Sometimes, tradition gets in the way of science.

Other plant pigments (new information)

Carotenoids are present in the antenna systems and reaction cores and in other locations. They include alpha and beta carotene, and xanthophylls: lutien, zeazanthin, violaxanthin, antheraxanthin and neozanthin. Carotenoids are involved in a number of processes including: Acting as antenna in the transfer of energy to PS II and PSI reaction centers; the xanthophyll cycle is used as an antenna or to shade PSII in plant photo-protection. Shading occurs in the blue and green part of the visible spectrum (Laisk 2014), to dissipate excess light energy, the blocking of free oxygen and organic radical, the quenching of chlorophyll excited states as well as singlet oxygen (Gitelson 2002).

The ratio of red light leaf absorption to blue light leaf absorption changes with actinic light intensity level (Bernacchi 2002). There is significant evidence to show that violazanthin converts to zeaxanthin as light levels increase, and that zeazanthin binds with the protein PsbS in a photoprotective role that shifts the absorption spectrum (Aspinall-O’Dea 2002). The xanthophyll cycle shields PSII reaction centers from intense blue light that can be especially harmful to PSII reaction centers (Laisk 2014). The xanthophyll cycle does not protect against intense red light. As a result, using actinic light sources with low blue intensity and high red intensity does not activate the xanthophyll cycle as it is in nature. In addition, zeaxanthin, in stoma guard cells, has been linked to stoma aperture size and appears to be mediated by blue light (Kinoshita 2001), (Ziegler 1998).

The function of anthocyanins in leaves, has been the subject of debate. They have been found most commonly in cell vacuoles but may be found in all plant tissues. There is evidence to show that they function as longer term photo-protective mechanisms, help serve in the protection of shade leaves from high intensity sun flecks, help provide protection against UV-B, function in antioxidant activity, act as attractors to animals for pollen and seed distribution, or act indirectly in signaling mechanisms involved in plant growth, and development, plant stress response, and gene expression. The affects can vary from one species to the next (Gould 2009).
Assessing plant physiology and plant stress with chlorophyll fluorescence

Chlorophyll fluorescence measurement has become a widely used technique for measuring plant stress, it is used for investigating the light reaction of photosynthesis, and its interaction with the dark reaction. It is important to use the correct measuring protocol for a specific type of plant stress as each protocol has limitations for a few specific types of plant stress measurement (Desk Top Plant Stress Guide). Some protocols will work for early detection of some types of plant stress, while on other types of plant stress, that same protocol may not work until the plant stress type is severe. The most used protocols, FV/FM, and Y(II), are fast tests that normally take only seconds. This allows the measurement of statistically significant plant populations in the field, or in the laboratory. The other factors that have made these techniques popular include light weight portable instrumentation, and the relatively inexpensive pricing of instrumentation.

Longer protocols also exist including: Quenching measuring protocols for the measurement of plant photo-protective mechanisms, chloroplast migration, state transitions, photoinhibiton, and the fraction of open reaction centers at a given actinic light level. It is also possible to create fluorescence light curves to measure plant responses to different steady state PAR light levels, and to use rapid light curves to measure plant responses to rapidly changing PAR (photosynthetically active radiation) light levels.

Chlorophyll fluorescence can also be used in combination with photosynthesis gas- exchange instrumentation to study both the light and dark reaction. This combination now allows the measurement of additional photosynthesis parameters that are unavailable without the combined use of gas-exchange and chlorophyll fluorescence. They include mesophyll conductance or g_m, and CO_2 at the sight of carboxilation or C_3 in the chloroplast. The combination is also useful for cold stress measurement where J or rETR values are about three times higher than expected when compared to gas exchange measurements (Desk top Plant Stress Guide). It is also useful in the study of C_3 plants under photorespiratory conditions found in plant drought stress, and in heat stress measurements (Desk top Plant Stress Guide).
Measuring fluorescence

The most common ways to measure chlorophyll fluorescence involve modulated fluorometers, continuous fluorometers and fluorometers that use ratios of fluorescence values.

FV/FM protocol

FV/FM is a normalized ratio that allows comparison of plant samples at a known dark adapted state. For this reason, and because the cost of instruments that just measure FV/FM are lower than instruments that measure multiple measuring protocols, FV/FM is the most used chlorophyll fluorescence measuring parameter in the world. FV/FM is measured with a modulated fluorometer and can be estimated with a continuous fluorometer. This is a fast test that requires adequate dark adaptation (Desk Top Plant Stress guide).

FV/FM = (FM – FO) / FM

FM is maximum fluorescence, or the peak fluorescent value from the Kautsky curve. It represents measurement of a sample after proper dark adaptation, when all available PSII reaction centers are in an oxidized, open condition. The sample is then exposed to a saturating light that closes, or chemically reduces, all available PSII reaction centers. With a saturation light, FM is the highest fluorescence value or an average of the highest values.

FO is minimum fluorescence. It represents fluorescence emitted from antennae before the any QA (quinone A) has been chemically reduced in the leaf. Using a modulated light fluorometer, a very weak modulated light, < 1 μmol, is used to excite this minimum fluorescence. It must be high enough to allow measurement, but low enough so that it does not drive the charge separation reaction of the light reaction. FO is then pre-photosynthetic fluorescence before any QA has been reduced (Zhu 2005). In continuous fluorometers, FO is estimated using linear regression analysis from the initial slope of the fluorescent increase.

It has been found that for many types of plant stress, the number of Q– non-reducing centers increase, raising FO, and reducing FV/FM. FM can also be reduced by larger plastoquinone pools and higher plasutoquinoe reoxidation constants (Zhu 2005). The maximum FV/FM value for a healthy plant under ideal conditions is between 0.79 and 0.83. for most species. Lower values indicate plant stress. While FV/FM may be used for all types of plant stress, it can best be used where the technique is capable of measuring plant stress at usable levels. For some types of plant stress, other chlorophyll fluorescence protocols and assays are available to measure (Desk Top Plant Stress Guide)

FV/FM was developed by Kitajima and Butler in 1975. Results have been shown to correlate well with photosynthesis measurements for many types of plant stress. (Desk Top Plant Stress Guide)

These are the graphic displays of the OS30p+ and the OS1p using the FV/FM measuring protocol. The OS30p+ is on the left and the OS1p is on the right. Saturation pulse duration time is the same for both, but the graphic display is set up to scale differently here.
Note: Many research reviewers like “overnight dark adaptation” even for FV/FM. It is recommended that you check with your target publications to comply with their views before experimental design.

**Y(II) protocol**

Y (II) also known as ΔF/FM’ , and $\Phi_{PSII}$  

$$Y (II) = (F_M' - F_S) / F_M'$$

This is a fast light adapted measurement taken only when the plant is at steady state photosynthesis. Developed in 1989 and 1990 by Bernard Genty, this test requires a modulated fluorometer. F_S is the chlorophyll fluorescence intensity value emitted by a plant at steady state photosynthesis. It is also known as F’. F_M’ is the maximum fluorescence measurement when a saturating light is applied to the leaf at steady state photosynthesis. Traditional views are that qT takes between 15 to 20 minutes at a fixed light level to reach steady state photosynthesis (Maxwell and Johnson 2000). With the new data regarding chlorophyll fluorescence and chloroplast migration, that time range may be extended from 20 minutes to 35 minutes at high light levels (Cazzaniga 2013). Sun leaves under a cloudless sky would be considered to be at steady state in the field.

Y (II) varies with PAR light intensity; therefore it is important to measure PAR with a meter near the leaf and at the same angle as the leaf, while it reached steady state.

Y(II) has been shown to correlate to photosynthesis measurements under many plant stress conditions. It has also been shown to be more sensitive to more types of plant stress or, in some cases, detect plant stress conditions earlier than Fv/Fm. For example Fv/Fm will only detect heat stress above 45°C, while Y(II) has been shown to detect plant heat stress at 35°C. (Haldiman P, 2004) (Desk top plant stress guide)

Y(II) works very well for most types of plant stress measurement in C4 plants, including drought stress. In C3 plants, photorespiration can create problems for drought stress measurement unless it is severe (Flexas 1999, 2000). In this case, a special assay, called the “Burke assay”, can be used to detect early drought or water stress. (Desk Top Plant Stress Guide).  (See the section on Loriaux 2013 F_M’ correction for important changes to Y(II) under high actinic light conditions.)

**rETR or J- Electron Transport Rate**  (new information)

rETR is relative electron transport rate and J is the equivalent value used by gas – exchange experts. In its basic form, it is an estimate of the number of electrons transported through photosystem II under steady state photosynthetic conditions. It has also been shown to correlate well to gas exchange photosynthesis measurements under many types of plant stress conditions.

This is a graphic display of the OS1p using the Y(II) measuring protocol.
rETR = Y(II) x 0.84 x 0.5 x PAR is a relative equation using average values.

or rETR = Y(II) x leaf absorptance x the PSII/PSI ratio x PAR (Photosynthetically Active Radiation). Because leaf absorptance and PSII/PSI ratios are, many times, unknown, it is common to use average values for these parameters. For example: Y(II) x 0.84 x 0.5 x PAR. For these reasons, Y(II) should be used for measurement instead of rETR when possible.

Leaf absorbance can vary for 0.70 to 0.90. Eichelman H. (2004). Leaf absorbance varies not only by species, but it can also vary by the plant type (C₃ or C₄), plant age, chlorophyll content, the amount of some types of plant, such as drought stress, Carter (1993), or if plants were subjected to some types of plant stress while growing (Baker 2008). It was recently found that leaf absorbance also changes with light intensity (Cazzaniga 2013, Dall’Osta 2014). At near saturating light intensities, chloroplasts migrate from the top of plant cells to the sides of the cell increasing leaf transmittance, and decreasing leaf absorbance.

The ratio of PSII reaction centers to PSI reaction centers can vary from 0.4 to 0.6 (Edwards GE. 1993), (Laisk A. 1996). C₄ plants tend to be in the ratio range of 0.4 PSII reaction centers, and C₃ plants tend to be closer to a 0.5 PSII ratio range. Eichelman (2004), Edwards (1993), Laisk (1996) & Laisk (2014) are good sources for information on individual species and conditions. Laisk 2014 provides up to date information on PSII/PSI ratio information. PSII/PSI ratio varies with species, plant type (C₃ or C₄), light quality during growing conditions, carbon deficiency (Zell 2010), (Anderson 1999) (Elgass 2011), recent light history due to photoinhibition and state transitions. (See the section on chloroplast migration and state transitions for more information on this subject, later in this document) (Laisk 1996), (Fan 2007) (Laisk 2014).

It has been stated that ETR or J should only be used for comparisons on leaves where the leaf absorptance and ratio of PSII to PSI reaction centers have been measured (Baker 2008). Leaf absorbance changes with age, some types of plant stress, chlorophyll content, growing conditions, actinic light intensity and light quality. (Cazzaniga 2013). (Bernacchi 2002) (Baker 2008). It has been common to measure leaf absorbance using an integrating sphere (Baker 2008), (Bernacchi 2002); however, estimates have been made using less exacting methods. For measuring the ratio of PSII to PSI, it has been common to use spectral analysis of samples at 77°K. Laisk (2014) and Peterson (2014) provide up dated methods of PSII/PSI ratio determination. (See the section on Loriaux 2013 Fₘ’ correction for important changes to Y(II) under high actinic light conditions.)

**Loriaux 2013 Fₘ’ correction**

Over the years, several researchers have found that it was impossible to completely close, or chemically reduce, all PSII reaction centers, under high actinic light or near saturating light conditions, even when using the most intense saturating light flash. This means that under intense actinic light conditions, Y(II) or ϕPSII, Fₘ’, and ETR are underestimated. According to Loriaux (2006), ETR or J can be underestimated by up to 41%, and Y(II) can be underestimated by up to 22%. As a result the Loriaux 2013 researchers, that included Bernard Genty, the developer of the quantum yield of PSII parameter, verified the issue, and developed a method for Fₘ’ correction.

While most believe it is possible to reduce or close all reaction centers in a properly dark adapted sample, with a relatively low amount of light, it has been found that in light adapted samples, with a high actinic light history, complete closure of all PSII reaction centers becomes problematic with, even the highest amounts of saturation light. It is thought that complete reduction of QA is prevented by fast turnover of the plastoquinone pools. (Margraph 1990, (Earl 2004) Loriaux 2006, Loriaux 2013).
The method was first developed by Loriaux 2006 in a poster, and later refined in a Loriaux 2013. It involved a multiple phases single saturation pulse with multiple light intensities, and the use of least squares linear regression analysis of the reciprocal of PAR light intensity, or Q, to determine the F_M’ fluorescence level, if an infinitely intense saturation pulse where used, but without causing damage to the plant.

The first saturation flash step, shown on the left below, is at 7,000 μmols for 0.30 seconds to saturate PSII. The saturation flash intensity is then ramped downward by 20%, making a large number of fluorescence measurements along the way, to 5,600 μmols. The final phase is at 7,000 μmols to check for saturation pulse NPQ. Recent studies have shown that optimal results occur for plants that have been tested with a first saturation flash at 7,000 μmols, a downward ramp in light intensity of 20% and a ramping rate less than 0.01 mol photons m^{-2}s^{-2} (Loriaux 2013).

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The graph on the right represents the Loriaux (2013) method for estimating F_M’ with an infinitely intense saturation flash. Least squares linear regression analysis of the reciprocal of PAR or Q values, measured at known saturation flash intensities, a down ramp of 20%, and a ramp rate that is less than 0.01 mol photons m^{-2}s^{-2} allow determination of the y intercept, which represents the machine fluorescence value with an infinite saturation flash.

![Graph showing machine fluorescence values](image)

The graphs above help explain the method described by the Loriaux, Burns, Welles, McDermitt, & Genty (2006) and expanded by Loriaux, Avenson, Welles, McDermitt, Eckles, Riensche, & Genty (2013).

Currently, all Opti-Sciences chlorophyll fluorometers that measure Y(II) and ETR use the Loriaux 2013 protocol include this option for measurement, along with standard square topped flashes. It is also available as an option for all quenching protocols, light curves and rapid light curves. Older OS1p models can be upgraded to Loriaux 2013 for free. For more information, contact Opti-Sciences Inc.
Quenching measurement protocols

There are two types of fluorescence quenching measurements, non-photochemical quenching and photochemical quenching. The uses of a modulated fluorometer is required for these measurements.

Photochemical quenching is a measure of the fraction of still open PSII reaction centers.

Non-photochemical quenching is a measure of heat dissipation impacted by photo-protection mechanisms, chloroplast migration, state transitions, and photoinhibition that can be affected by different types of plant stress. Most of parameters used for measuring quenching must be made at steady state photosynthesis to be valid by definition; however, Y(NO) in the lake model does not need to be at steady state. (OSI quenching application note)

When making quenching measurements, samples should only be compared if they have the same F_v/F_m values, since F_v/F_m is the measuring reference for quenching values (Baker 2008).

Lake model and puddle model quenching parameters.

Understanding of the organization of antennae and reaction centers has changed over the years. It is now understood that a single antennae does not link only to a single reaction center as was previously described in the puddle model. Current evidence indicates that reaction centers are connected with shared antennae in terrestrial plants. q_P, the parameter that has been used in the past to represent the fraction of PSII reaction centers that are open, is a puddle model parameter. Dave Kramer (2004) has come up with a set of fluorescence parameters that represent the newer shared antennae paradigm called the lake model.

These three graphs represent the display screens from the OS5p using three different quenching protocols. The graph on the left is using the Hendrickson lake model quenching protocol with NPQ resurrected from the puddle model. The center display shows the Kramer lake model protocol and the display on the right shows the puddle model protocol with quenching relaxation protocol.
Others have also come up with more simplified equations that eliminate the need for the measurement of Fo’ and approximate the measurements made by Kramer. Fo’ is a controversial parameter in some circles.

Hendrickson’s (2004) work offered an alternative lake model solution with Y(NPQ) measurements that are consistently and only marginally lower values than Kramer’s work, and Y(NO) measurements that are consistently and marginally lower except at high light levels and low temperatures than Kramer’s work. He speculates that the differences in values between Kramer and his own were possibly due to the difficulties in making Fo’ (or Fod) measurements. Furthermore, Hendrickson does not provide a photochemical quenching parameter like qL to estimate the fraction of open PSII centers.

From Hendrickson’s work, earlier works by Cailly (1996), and Genty (1989, 1990), Klughammer and Schreiber derive simplified equations that allow NPQ to be reconciled with the lake model. Since there is a large volume of work that has been done investigating large numbers of plant species and plant mechanisms using NPQ, it allows the transition from puddle model to lake model measurement to occur in less painful way. Furthermore Hendickson’s equations provide lake model parameters without the use of the controversial parameter Fo’. Today, all of these methods are still in use.

**Kramer’s lake model equations:**

\[ Y(II) = \frac{(F_{M'} - F_S)}{F_{M'}} \text{ or } \frac{\Delta F_{M'}}{F_{M'}} \]

\[ Y(NO) = \frac{F_S}{F_M} \text{ or } \frac{F'}{F_M} \]

\[ Y(NPQ) = \frac{F_S}{F_{M'}} - Y(NO) \text{ or } \frac{F'}{F_{M'}} - Y(NO) \]

\[ q_L = \frac{(F_{M'} - F_S)}{(F_{M'} - F_O)}(F_{O'}/F_S) \text{ or } q_L = q_P(F_{O'}/F_S) \]

**Hendrickson’s lake model equations:**

\[ Y(II) = \frac{(F_{M'} - F_S)}{F_{M'}} \text{ or } \frac{\Delta F_{M'}}{F_{M'}} \]

\[ Y(NO) = \frac{F_S}{F_M} \text{ or } \frac{F'}{F_M} \]

\[ Y(NPQ) = \frac{F_S}{F_{M'}} - Y(NO) \text{ or } \frac{F'}{F_{M'}} - Y(NO) \]

**Klughammer and Schreiber’s lake model NPQ, determined from Hendrickson’s equations.**

\[ NPQ = \frac{Y(NPQ)}{Y(NO)} \text{ or } NPQ = \frac{(F_M - F_{M'})}{F_{M'}} \]

**Puddle model equations:**

\[ q_P = \frac{(F_{M'} - F_S)/(F_{M'} - F_O)}{(F_{M'} - F_O)} \text{ or } q_P = \frac{F_{M'} - F'}{(F_{M'} - F_O)} \text{ Above } 0.4, F_O \text{ should replace } F_O \]

\[ q_N = 1 - \frac{(F_{M'} - F_O)}{(F_{M} - F_O)} \text{ Above } 0.4, F_O \text{ or } F_{O'} \text{ should replace } F_O \]

\[ NPQ = \frac{(F_M - F_{M'})}{F_{M'}} \text{ or } NPQ = \frac{(F_M - F_{M'})}{F_{M'}} \]

**Quenching relaxation equations for the Lake or puddle model**
NPQ = qE+qT+qI (traditional) At high light level, one could now consider qM as part of NPQ instead of qT, although it is technically not considered a non-photochemical quenching mechanism. It is also true that qT is also not technically a non-photochemical quenching mechanism.

\[ qE = \frac{(F_{ME}-F_{M'})}{(F_M-F_{M'})} \]  
\[ F_{ME} \] is the relaxation saturation value at four to ten minutes in the dark. \( F_{M'} \) is the last light adapted \( F_{M'} \) value at steady state fluorescence. Field plants require the longer times to measure \( qE \). Times of up to ten minutes have been reported.

\[ qT = \frac{(F_{MT}-F_{ME})}{(F_{M}-F_{M'})} \]  
\[ F_{ME} \] is the relaxation saturation value at twenty minutes in the dark.

\[ qM = \frac{(F_{MM}-F_{ME})}{(F_{M}-F_{M'})} \]  
\[ F_{MM} \] is the relaxation saturation value that takes more than a ten minutes to times of from twenty to 35 minutes in the dark. \( qM \) is the intermediate chlorophyll fluorescence change due to chloroplast migration

\[ qI = \frac{(F_{M}-F_{MT})}{(F_{M}-F_{M'})} \]  
Relaxation of \( qI \) starts at about forty minutes and can take up to sixty hours. \( qI \) can be determined from the dark adapted \( F_{M} \) measurement and the saturation pulse after \( qM \) or in some cases \( qT \).

Since NPQ has been resurrected for the lake model, the quenching parameters \( qE \), \( qT \), and \( qI \) are also valid in the lake model.

**Measurement of \( qE \), \( qM \), \( qT \) and \( qI \)**

\( qE \), \( qM \), \( qT \) and \( qI \) require a quenching relaxation measuring protocol that is only available on the most advanced chlorophyll fluorometers. A stable light source is required to achieve a reliable steady state photosynthesis level. These measurements take time. It is common to dark adapt overnight, and then expose the leaf to a stable light source at a specific intensity for twenty to 35 minutes, the time required to reach steady state photosynthesis in many plants. It then requires the use of saturation pulses in the dark to measure quenching relaxation for another twenty to thirty five minutes while \( qE \), \( qM \), or \( qT \) relaxes. A modulated light is also used (see the quenching app note for more details).

**Quenching parameter definitions, advantages, and limitations:**

**Puddle model**

NPQ (puddle model and Henrickson lake model parameter) is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. It provides an estimate of quenching without knowledge of \( F_{O'} \). The advantage of NPQ over \( qN \) depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of nonphotochemical quenching associated with \( qN \) values lower than 0.6. The range of NPQ is affected by \( \Delta pH \) of the thylakoid lumen which is an important aspect of photosynthetic regulation, chloroplast migration, state transitions and photoinhibition. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000). (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001).

\( qN \) (puddle model quenching protocol parameter) is similar to NPQ but requires \( F_{o} \) or \( F_{o'} \) in the calculation. \( qN \) is defined as the coefficient of non-photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (Fv) and not the minimal fluorescence (Fo). In cases where \( qN \) is greater than 0.4 this may not be a good assumption. When \( qN \) is above 0.4, \( F_{o'} \) (or Fod) should replace \( F_{o} \) in \( qN \) equations. \( qN \) is less sensitive than NPQ at higher values (Maxwell and Johnson 2000). By using the Far-Red source after actinic illumination is turned off, the PSII acceptors re-oxidized and PSI is reduced. A new \( F_{o'} \) value is measured and used for
corrections to the quenching coefficients. Numbers range from zero to one. (puddle model) (Van Kooten & Snel, 1990)

**qP** *(puddle model protocol parameter)* is the quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. qP is defined as the coefficients of photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (Fv) and not the minimal fluorescence (Fo). In cases where qN is greater than 0.4 this may not be a good assumption. When qN is above 0.4, Fo' (or Fod) should replace Fo in qP equations. By using the Far-Red source for post illumination, the PSII acceptors may be re-oxidized through the illumination affect on PSI. A new Fod value may be measured and used for corrections to the quenching coefficients. This assumes the PSI acceptors are properly activated, which may not be the case in a dark adapted sample. Therefore, the Fod determination should be done after induction of photosynthesis has been done for several minutes. Numbers range from zero to one. (Puddle model) (Van Kooten & Snel, 1990)

**qE** *(puddle model or Hendrickson lake model quenching relaxation protocol parameter)* is the quenching parameter that represents the photo-protective mechanisms in the leaf that allow rapid compensation for changes in light levels due to cloud cover and increased light intensity. It is directly related to ΔpH of the thylakoid lumen and the xanthophyll cycle. (Muller P., Xiao-Ping L., Niyogi K. 2001) This process is completed in two to four minutes after an actinic light is turned on. (Lichtenthaler 1999). It is delineated from NPQ by using a quenching relaxation method. Some researchers in the past have also divided qN into qE, qT, and qi instead of NPQ (Lichtenthaler 1999) The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008).

**qT** *(puddle model or Hendrickson lake model quenching relaxation protocol parameter)* is not true quenching. Instead, the parameter represents state 1 and state 2 transitions. This value is negligible in higher plants at high light levels but may be substantial at low light levels (Lichtenthaler 1999) (Baker 2008). According to Ruban (2008) state transitions require between fifteen and twenty minutes to complete. It can be delineated from NPQ by using a quenching relaxation method (Muller P., Xiao-Ping L., Niyogi K. 2001). For more information on state transitions, and how they affect fluorescence measurement contact Opti-Sciences for the application note on state transitions. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008). *There is now strong evidence that qT does not exist in most land plants at high light intensities. The evidence points to qM or chloroplast migration and the resulting reduction in leaf absorptance as the source of qT.* (Cazzaniga 2013).

**qM – due to chloroplast migration**

Cazzaniga S, Oslo L.D., Kong S-G., Wada M., Bassi R., (2013) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change, previously thought to be the result of state transitions and chronic photoinhibition, or more recently, thought to be a longer lasting xanthophyll cycle process, was caused, by *chloroplast migration*. They found, as others have, that under high light levels, chloroplasts move from the top of cells to the sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research concludes that the cause of qM is a decrease in light photon absorption which creates lower fluorescence yield, rather that a true quenching process. This is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity change mirrored the previously used qT and chronic photoinhibition, up to 30 minutes, but extended up to 35 minutes with some mutant plants. Chloroplast migration has been known and studied for a while, and it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga paper is the first to name chloroplast migration as the source of the qT acute photoinhibition or qZ fluorescence change.
Researchers found that high white actinic light and intense blue light induced chloroplast migration as found in nature. However, intense red light did not induce chloroplast migration as found in nature. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa T. 2001). Arabidopsis mutants that were devoid of $q_E$ (npq4) were tested and Arabidopsis mutants devoid of $q_E$ and chloroplast migration (npq4 photo2) were also tested along with other mutants. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutant that covered the remaining spectrum of mechanisms that affected chlorophyll fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, and state transitions as sources of $q_M$.

In regard to $q_T$ being replaced by $q_Z$, it was found that by using mutants devoid of $q_E$ and zeaxanthin, that the magnitude of $q_M$ did not change but the recovery time in the dark was longer. Plants were grown at 150 $\mu$mol photons m$^{-2}$ s$^{-1}$, and tested at 400 $\mu$mol photons m$^{-2}$ s$^{-1}$, 800 $\mu$mol photons m$^{-2}$ s$^{-1}$, and 1,200 $\mu$mol photons m$^{-2}$ s$^{-1}$. The adjustment time for $q_M$ was from 20 to 30 minutes but ranged up to 35 minutes for some mutants.

The Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) observation that the stn7 mutant, devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of $q_T$ in Arabidopsis.

Chloroplast migration as found in nature, requires a white actinic light or an intense blue light to function correctly. Red light or an intense red light with a lower intensity blue light will cause measuring artifacts at high light intensities (Cazzaniga 2013).

$q_I$ - puddle model or Hendrickson lake model quenching relaxation protocol parameter is the quenching parameter that represents photo-inhibition and photo-damage. (Puddle model) (Muller P., Xiao-Ping L., Niyogi K. 2001) According to Lichtenthaler (1999, 2004) chronic photoinhibition starts to relax after forty minutes in the dark and may take up to sixty hours. It can be delineated from NPQ by using a quenching relaxation method. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008). Photoinhibition targets PSII reaction centers. It has the affect of balancing ATP output by PSII and NADP output by PSI. With a low light history, PSII reaction centers outnumber PSI reaction centers (Laisk 2014). Until recently, acute photoinhibition was considered part of $q_I$. After Cazzaniga 2013, and Dall’Osta 2014, acute photoinhibition changes should be attributed to $q_M$ or chloroplast migration.
Kramer lake model quenching parameters

Y(NPQ) (lake model quenching parameter) It represents heat dissipation related to all photo-protective mechanisms also called regulated heat dissipation. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004) (Klughammer and Schreiber 2008). A low Y(NPQ) at high light levels is an indication of sub-optimal photoprotective mechanisms. (Klughammer and Schreiber 2008).

Y(NO) (lake model quenching parameter) It represents all other components of non-photochemical quenching that are not photo-protective. They include non-radiative decay, and fluorescence. Part of Y(NO) includes photoinhibition (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004). Klughammer and Schreiber define Y(NO) as the “fraction of energy that is passively dissipated in the form of heat and fluorescence mainly due to closed PSII reaction centers”. Hendrickson calls Y(NO) constitutive heat dissipation. A high Y(NO) value after dark adaptation is an indication of photodamage. (Klughammer and Schreiber 2008).

qL (Kramer lake model quenching parameter) It represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004).

Y(II) = (lake model) quantum yield of photosynthetic energy. The equation is the same as for Y, or $\frac{\Delta F}{F_m'}$.

Hendrickson lake model quenching parameters (these are approximations of Kramer values without measuring $F_0'$). NPQ can now be resurrected to the lake model using Hendrickson equations (Klughammer and Schreiber 2008).

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Y(II) = (lake model) quantum yield of photosynthetic energy. The equation is the same as for Y, or $\frac{\Delta F}{F_m'}$. ($F_m' - F_s$) / $F_m'$ or ($F_m' - F$) / $F_m'$

NPQ (puddle model and Hendrickson lake model parameter resurrected to Hendrickson’s lake model by Klughammer.) is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. It provides an estimate of quenching without knowledge of $F_0'$. The advantage of NPQ depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of nonphotochemical quenching associated with low values. The range of NPQ is affected by $\Delta p$H of the thylakoid lumen, and the xanthophyll cycle which are an important aspect of
photosynthetic regulation. Chloroplast migration, state transitions and photoinhibition also affect NPQ. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000). (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001). NPQ is by far, the most used quenching parameter. As a result, there are a large number of published papers that can be referenced for most applications.

**Light Curves**

*Light Curves.* – These are protocols that step through different light levels to find the Y(II), rETR, and other values at different PAR levels. Measurement is only made after the plant has reached steady state photosynthesis. They can start low and move higher or start high and go lower. It takes between 15 minutes to 20 minutes at a given light level to reach steady state photosynthesis (Maxwell and Johnson 2000). However, new research by Cazzaniga 2013 and Dall’Osto 2014 indicate that it can take between 20 and 30 minutes for chloroplast migration to occur in wild plants and up to 35 minutes in some mutants. Therefore, steady state requires times of 20 to 30 minutes, in most plants, for steady state to occur, due to chloroplast migration.

Plants grown in low light conditions are structurally different than plants grown under high light condition. Plants grown under low light conditions will saturate at much lower PAR light (photosynthetically active radiation) intensities.

This is a light curve graph from the OS5p
**Rapid light curves** – Almost all other light adapted chlorophyll fluorescence measuring parameters require steady state photosynthesis, by definition, to provide reliable results except Y(NO). Rapid light curves are an attempt to provide measuring solutions for plant samples that are under variable light conditions (Ralph 2005). Plants that are under other plant canopies, and aquatic plants are subject to changing light conditions. With this type of test, plants are subjected to stepped PAR actinic light conditions much like light curves, but for very short period of time. It is common to use ten second steps, but times up to minutes have been used. It is also common to use momentary dark adaptation before starting the RLC, but longer times have been used. (Ralph 2005), (Rascher U. 2000).

Results vary with the time of day. Research shows that RLCs correlate well with rubisco activity under changing light conditions. (MacIntyre H. L 1996, 1997) The parameters provided are, rETR\textsubscript{MAX} - relative maximum electron transport rate, $\alpha$ is the initial slope of the line at low PAR values, $I_K$ is the point of minimum saturation level = rETR\textsubscript{MAX} / $\alpha$, and $I_M$ is the rETR\textsubscript{MAX} PAR level. It is common to use curve fitting software to graph the protocol and determine the values. (Application note on RLC)

Various curve fitting schemes have been used. We use Eilers and Peters curve fitting.

OS1p Rapid Light Curves. Eilers and Peeters curve fitting software algorithms are resident on the instrument for curve display.
Ratio fluorescence and ratio fluorometers

Ratio fluorescence has been explored to further understand plant physiology, and extend the uses of chlorophyll fluorescence into areas that have proved difficult to measure in the past. Nitrogen stress has been a particularly difficult stress to measure, at usable levels, using chlorophyll fluorescence. However, ratio chlorophyll fluorescence has shown great success in this area. Most solutions have been relatively expensive in the past, but now, the CCM-300 ratio fluorometer, using the Gitelson protocol, offers a cost effective solution to nitrogen stress, and chlorophyll content.

According to Neil Baker, chlorophyll fluorescence is not normally capable of measuring nitrogen plant stress until it is severe, and sulfur plant stress until starvation levels have been reached. (Baker 2004) Several research groups have worked on various fluorescent ratios in an attempt to measure chlorophyll content, that can be used for nitrogen and sulfur stress, measure general plant stress, or other specific types of plant functions. Researchers that include Gitelson, Lichtenthaler, Buschmann, Sampson and Cerovic have spent a great deal of time working in this area.

Using fluorescence emission ratios of multiple fluorescence excitation wavelengths, and ratios of different fluorescence emission wavelengths using the same excitation wavelength have been used, with various results.

Some of the ratios have been successful. In 2000, Samson used two excitation wavelengths and one emission wavelength to measure nitrogen stress at effective levels. The ratio was called FEX365/FEX440. Nitrogen Stress in plants was determined by the ratio of UV excited and blue excited far red fluorescence. Unlike leaf absorption techniques used for nitrogen testing, nitrogen stress can be distinguished from sulfur stress with this measurement (Sampson 2000). FRFex360/FRFex440 measures the concentration of UV absorbing compounds in the leaf epidermis which are higher in nitrogen stressed plants.

In 1999, Gitelson, Lichtenthaler, and Buschmann developed a refined technique from earlier fluorescent ratios that allowed a maximum reliable chlorophyll content measuring range. By limiting the emission spectrum of red fluorescence, the reliable measuring range of chlorophyll content was more than doubled compared to leaf absorption techniques that have been commonly used for nutrient plant stress measurement and nitrogen management. The Gitelson emission fluorescence ratio was F735/F700. It offered several advantages. It could be used on very small leaves, conifers, grasses, fruit, stems, petioles, moss on rocks or recently germinated Arabidopsis leaves. It also allowed reliable direct readout of chlorophyll content in mg/m^2 (Gitelson 1999).

Gitelson, Cerovic and Buschmann have worked with various ratios in an attempt to determine everything from plant stress to anthocyanin content.
These are graphs to show how the CCM-300 chlorophyll content meter works, using the Gitelson protocol for ratio chlorophyll fluorescence. The sample is exited using blue light, and the ratio of emission fluorescence is measured, along with direct readout in chlorophyll content using the formula from Gitelson 1999.

This is the CCM-300, ratio fluorescence, chlorophyll content meter measuring a white pine needle.
The Desk Top Plant Stress guide, and the application notes mentioned in this discussion can be found at: www.optisci.com

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Opti-Sciences (2012) Quenching application note www.optisci.com


External links

Opti-Sciences (2013) Quenching application note www.optisci.com
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