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Variable Chlorophyll Fluorescence – Overview (2013)

Chlorophyll absorbs light most effectively in the red and blue parts of the visible spectrum. Chlorophyll fluorescence is light that is re-emitted at a longer wavelength after being absorbed by chlorophyll molecules at a shorter wavelengths. Variable chlorophyll fluorescence is only observed in chlorophyll “a” in photosystem II. By measuring the intensity and nature of *variable* chlorophyll fluorescence, and 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, correlation with photosynthesis carbon assimilation, and measurement of most types of plant stress at usable levels (Baker 2004). As stated earlier, the sole origin of variable chlorophyll fluorescence is chlorophyll “a” in photosystem II (Zhu 2005). 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)

Photosynthesis is comprised of a light reaction and a dark reaction. The light reaction converts light energy into chemical energy that can be used in the dark reaction. 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 (Rosequist and van Kooten). 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 (Schreiber 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 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 and C₄ plants (Desk Top Plant Stress Guide).

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. (Govindjee 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 antenna are tightly bound chemically and adjacent to the reaction center. Peripheral antenna are near the core and 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 D_1 and D_2 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 D_1D_2 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 antenna 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_0 , or F_v/F_M (Govindjee 1978). There has been no fluorescence observed in chlorophyll "b" when energy transfer to chlorophyll "a" is normal. (Govindjee 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 Q_A . (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_0 , 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 A_0 molecule in photosystem I are the only steps where light energy is converted into chemical energy (Zhu 2005).

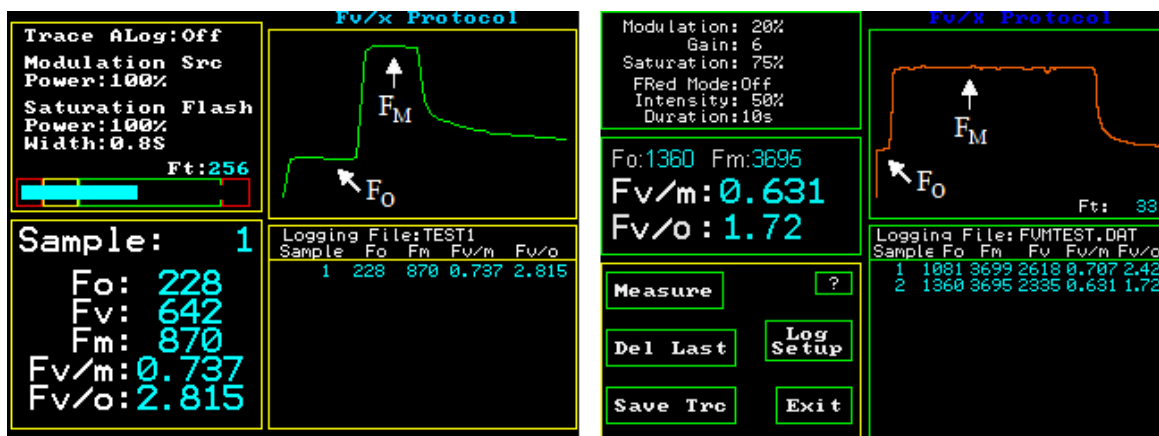
At charge separation in photosystem II, an electron is transferred from P_{680} , the primary electron donor, to the primary electron acceptor, pheophytin. The chemical process for charge separation is shown in diagram

A. During this process, an electron is added from Tyrozine, Y_Z , generated from the Oxygen Evolving Complex, through the Mehler 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 Q_A . Additional sources of minimum fluorescence also include Photosystem I, and PSII Q_B^- non-reducing reaction centers (Zhu 2005), (Opti-Sciences F_V/F_M checklist application note).

The rise of variable chlorophyll fluorescence starts at charge separation and continues through the reduction of Q_A , Q_B , and the reduction of the Plastoquinone Pool. Evidence shows that the rise ends with the re-oxidation of PQH_2 (Plastoquinol) to PQ (plastoquinone) by the Cytochrome b_6f complex. Maximum variable fluorescence or F_M , occurs when $Q_A Q_B^{2-}$ and PQH_2 are at a maximum (Zhu 2005). The height of F_M is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH_2 to PQ. A higher, k_{ox} , reoxidation constant, or a larger plastoquinone pool reduces the F_M value (Zhu 2005).



Measuring screens from the OS30p+ and the OS1p

F_0 represents minimum chlorophyll fluorescence before any Q_A has been reduced.

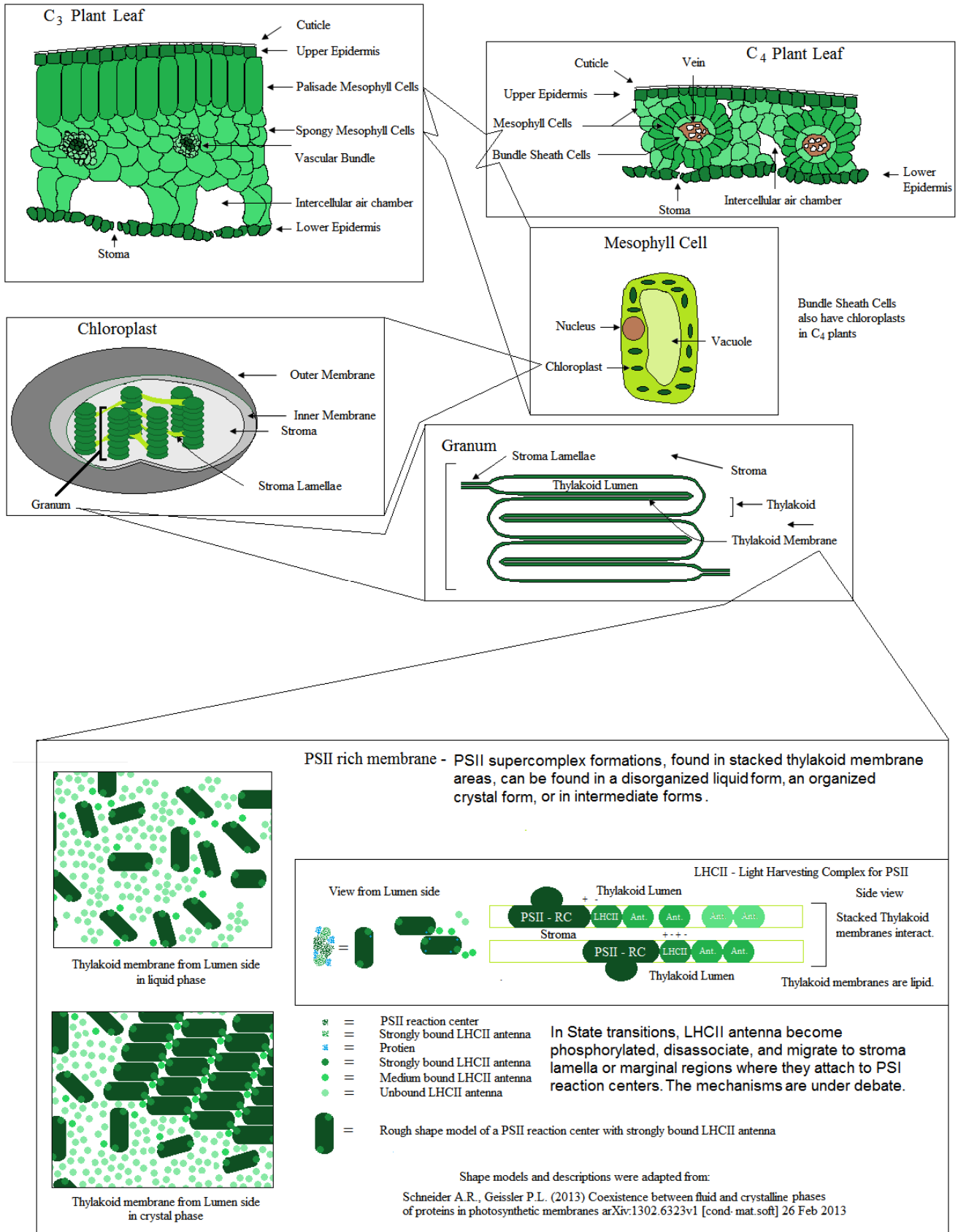
F_M is maximum variable chlorophyll fluorescence when $Q_A Q_B^{2-}$ and PQH_2 are at a maximum.

F_0 is affected by the number of the number of Q_B^- non-reducing reaction centers. Higher numbers raise F_0 . It is also affected by the ratio of PSII to PSI reaction centers. In C_4 plants, where the ratio of PSI reaction centers is higher than in C_3 plants, PSI non-variable fluorescence can represent up to 50% of F_0 (Shreiber 2004). It is also affected by dark adaptation status and setting the modulated light correctly. PSI gives off a low non-variable chlorophyll fluorescence.

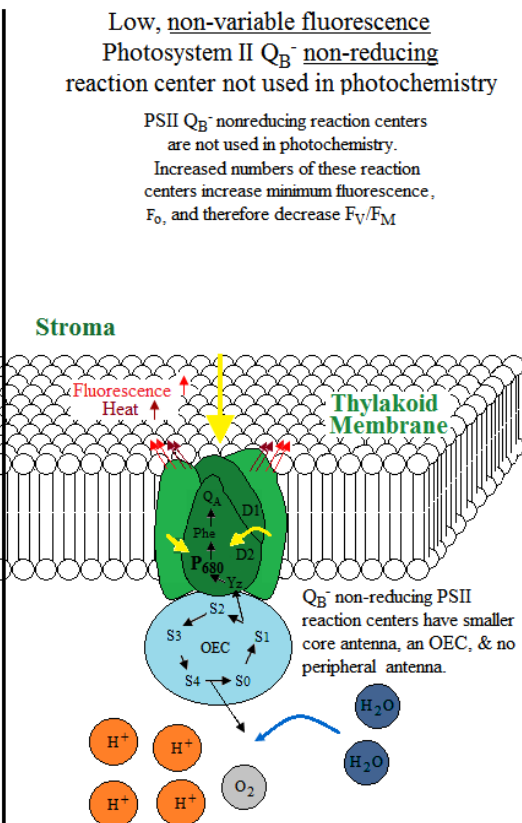
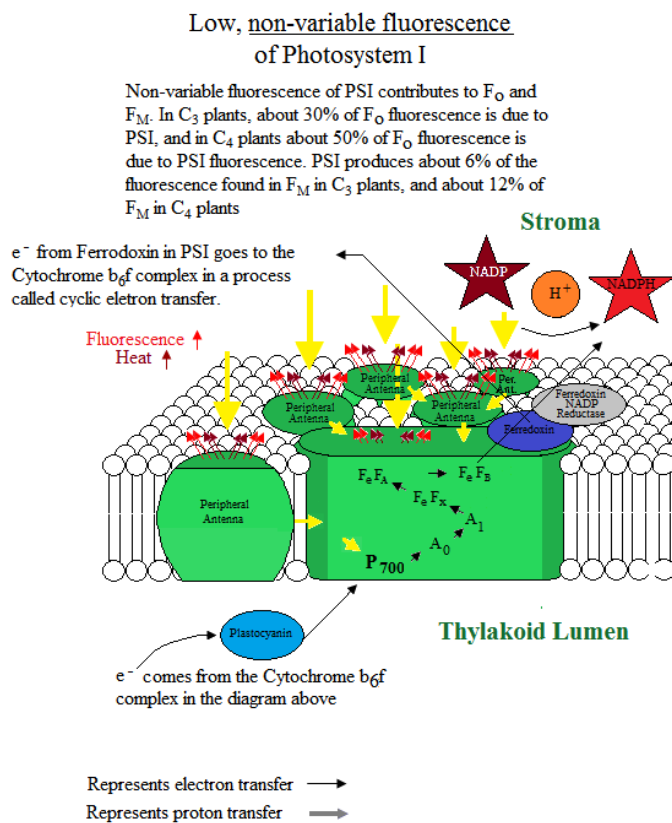
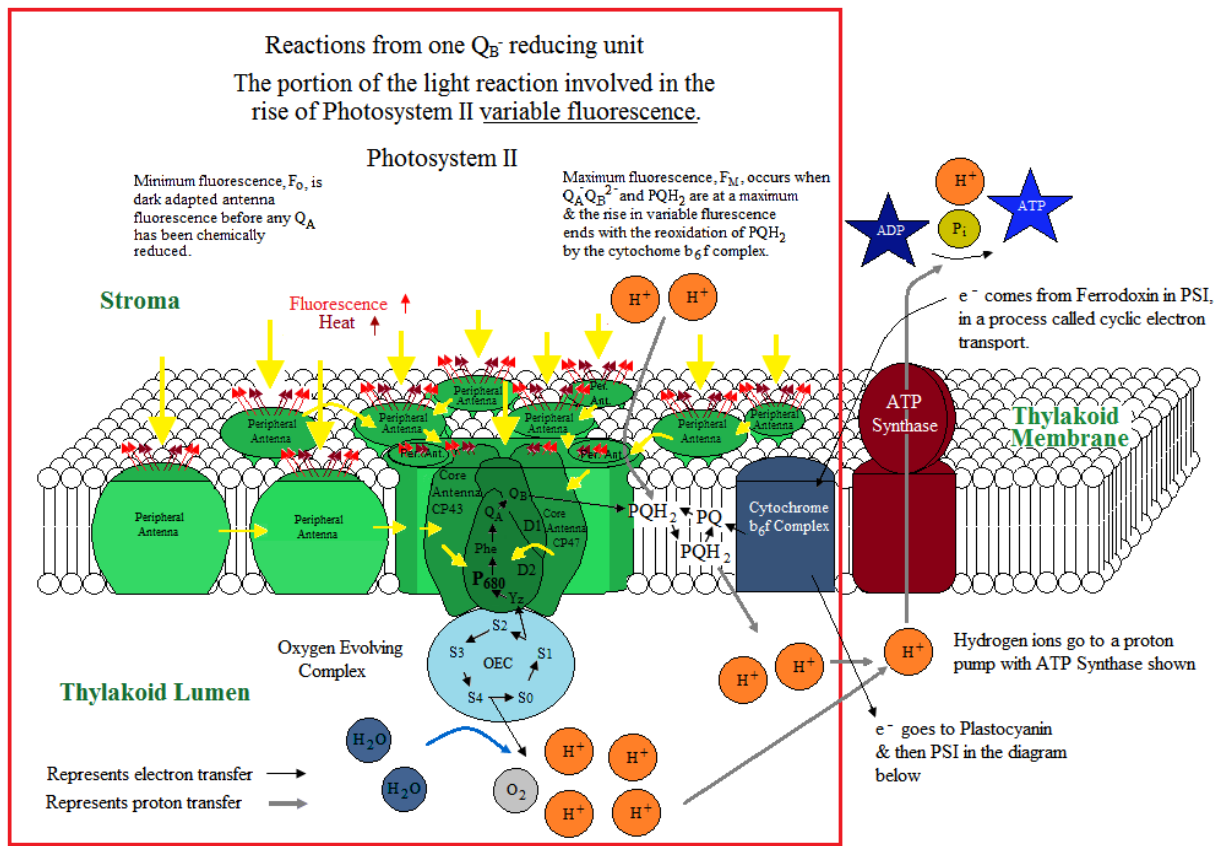
The height of F_M is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH_2 to PQ. A higher, k_{ox} , reoxidation constant, or a larger plastoquinone pool reduces the F_M value (Zhu 2005). It is also affected by using a proper dark adapted state to relax the xanthophyll cycle, ΔpH of the thylakoid lumen, and state transitions. In addition it can also be affected by light history as it applies to acute photoinhibition and chronic photoinhibition (Lichtenthaler 2004). Acute photoinhibition, caused by high light levels for an hour or two, relaxes or repairs in 30 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 (Lichtenthaler 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_V/F_M along with recommendations and references regarding specific types of plant stress.

(Diagram B)

Drill down diagram of variable and non-variable chlorophyll fluorescence



(Diagram C) This is a drill down diagram of the mechanisms that affect variable chlorophyll fluorescence. It designed as a more visual representation of the most accepted understanding of the sources of chlorophyll fluorescence and variable chlorophyll fluorescence. Chlorophyll fluorescence generating sources for Photosystem II were taken from Zhu 2005, & 2012. Photosystem I information was taken from Schreiber (2004)



(Diagram D)

D_1 and D_2 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)

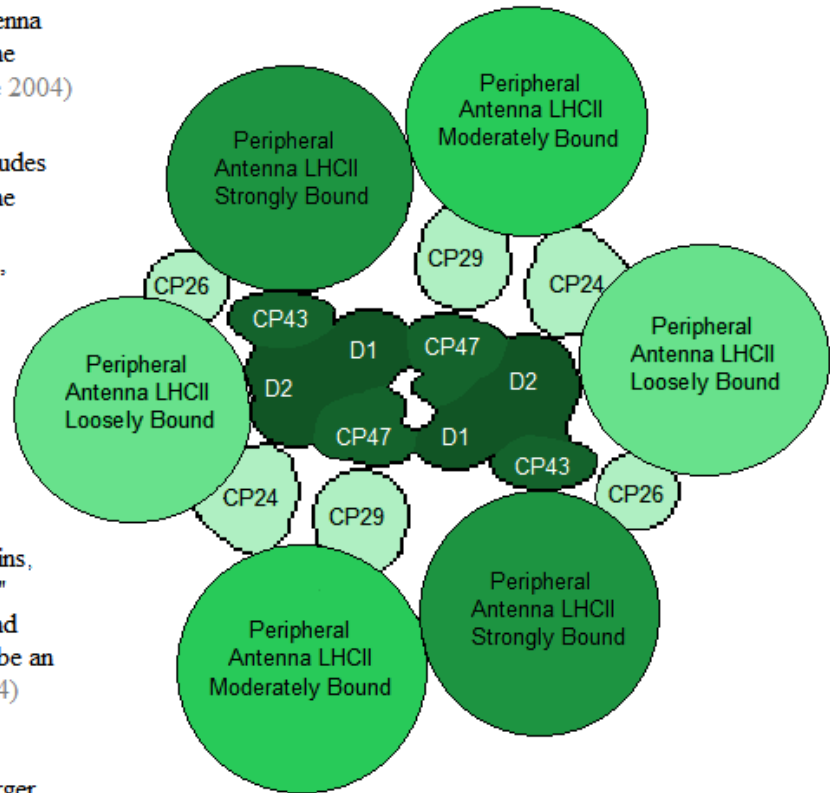
CP43 and CP47 are chlorophyll-protein core antenna complexes that are adjacent and associated with the D_1D_2 P680 PSII reaction center. (van Grondelle 2004)

X-ray diffraction shows that the PSII core that includes the D_1D_2 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)

LHCII, 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 LHCII 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)

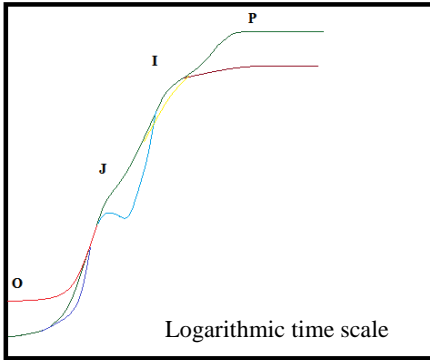
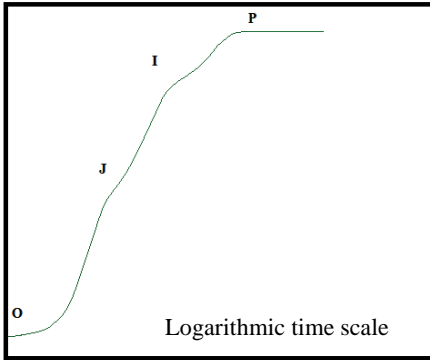


This is a constructed image of a photosystem II -LHCII supercomplex adapted from van Grodell 2004, and Boekema 1999.

Top view

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.

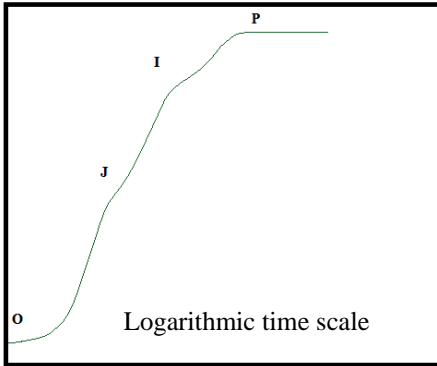


O – is commonly measured at 20 μsec . after the start of actinic illumination in continuous fluorometers, and is not equal to F_O measured by modulated fluorometers. Continuous fluorometers use linear regression analysis to estimate F_O , or minimum fluorescence in a dark adapted state, before any Q_A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

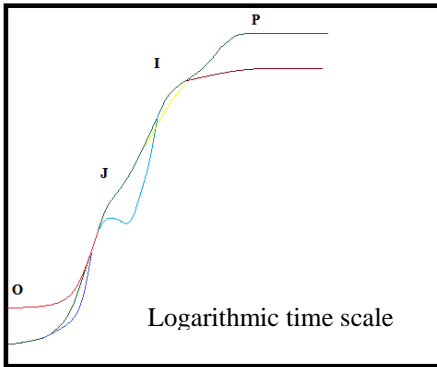
The height of both O and F_O are affected by the ratio of the size of peripheral antenna to core antenna. A larger ratio of peripheral antenna causes O and F_O to be lower.

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 Q_A . J represents maximum values for $Q_A Q_B^-$ and $Q_A^- Q_B^-$. 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 $Q_A Q_B^{2-}$ chemical equation that ends at P with a maximum for $Q_A^- Q_B^{2-}$. If properly dark adapted, the J to I rise starts with the ratio of $Q_B : Q_B^- = 1:0$ and ends with the ratio at 0:1. The dark adapted ratio prior to light exposure of $Q_B : Q_B^-$ 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 $Q_A^- Q_B^{2-}$, & 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. 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. (Vredenberg 2004)

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 3,500 μmol for the Strasser protocol and up to 6,000 μmol 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, 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 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 red and blue actinic light. The apertures of plant stoma are mediated by blue actinic light (Kinoshita 2001). The absorption ratio of red to blue light changes with light intensity (Bernacchi 2002).

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 other mechanisms, that are slower reacting, that 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 ΔPh 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 light levels, there is more non-photochemical quenching. This process takes about four minutes in greenhouse plants (Lichtenthaler 2004), but it can take up to seven minutes in field plants (Baker 2008). q_E is a parameter that is used to measure the non-photochemical quenching photoprotective mechanisms. This parameter is used in conjunction with quenching relaxation protocols (Muller 2001). q_L from the lake model and q_P 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 mechanism that continues to lower fluorescence output after the initial fluorescence rise. A mechanism called state transitions continue to adjust for between for fifteen to twenty minutes to finally reach “steady state photosynthesis” at a specific actinic light level. (Maxwell and Johnson 2000).

State transitions

State transitions, are thought to be a low light survival mechanism. This process can take between fifteen to twenty minutes at a fixed light level in field plants. It therefore overlaps somewhat with photoprotection mechanisms in q_E . (Ruban, Johnson 2009) (Maxwell and Johnson 2000). They act to balance light distribution between the two photosystems in an attempt to utilize all available light. State transitions are a larger component of light adapted fluorescence measurements taken at low light levels than at higher light levels. Lichtenthaler found that state transition adjustment of fluorescence levels represented 67% of non-photochemical quenching at 10 μmols PAR actinic levels, and only 5% of non-photochemical quenching at 2000 μmols PAR actinic levels, on the plants tested (Lichtenthaler 1999). q_T is the parameter used to measure the effects of state transitions (Muller 2001), (Ruban, Johnson 2009). This parameter is also part of quenching relaxation protocols. Photophosphorylation changes the affinity of some of these antenna from photosystem II to photosystem I. This causes some of these complexes to *detach*, *migrate* to Photosystem I and serve as antennae for Photosystem I. When the plastoquinone pool is more oxidized, it activates a second enzyme to dephosphorylate these transient antennae and cause migration to PSII reaction centers. Kinase enzymes that are associated with phosphorylation and the phosphatase enzyme is associated with dephosphorylation (Ruban, Johnson 2009) (Tikkanen 2011). As a result, the emitted chlorophyll fluorescence drops until steady state photosynthesis is reached. The initial rise in fluorescence levels, in land plants, can take from 0.3 seconds to 1.5 seconds. Subsequently, fluorescence decreases due to photochemical quenching, non-photochemical quenching, and state transitions can occur for between 15 to 20 minutes, until steady state photosynthesis is reached (Maxwell and Johnson 2000). While state transitions

are not really a form of non-photochemical quenching, they are both reflected in NPQ and q_N measuring values at steady state photosynthesis. (Muller 2001).

Photoinhibition

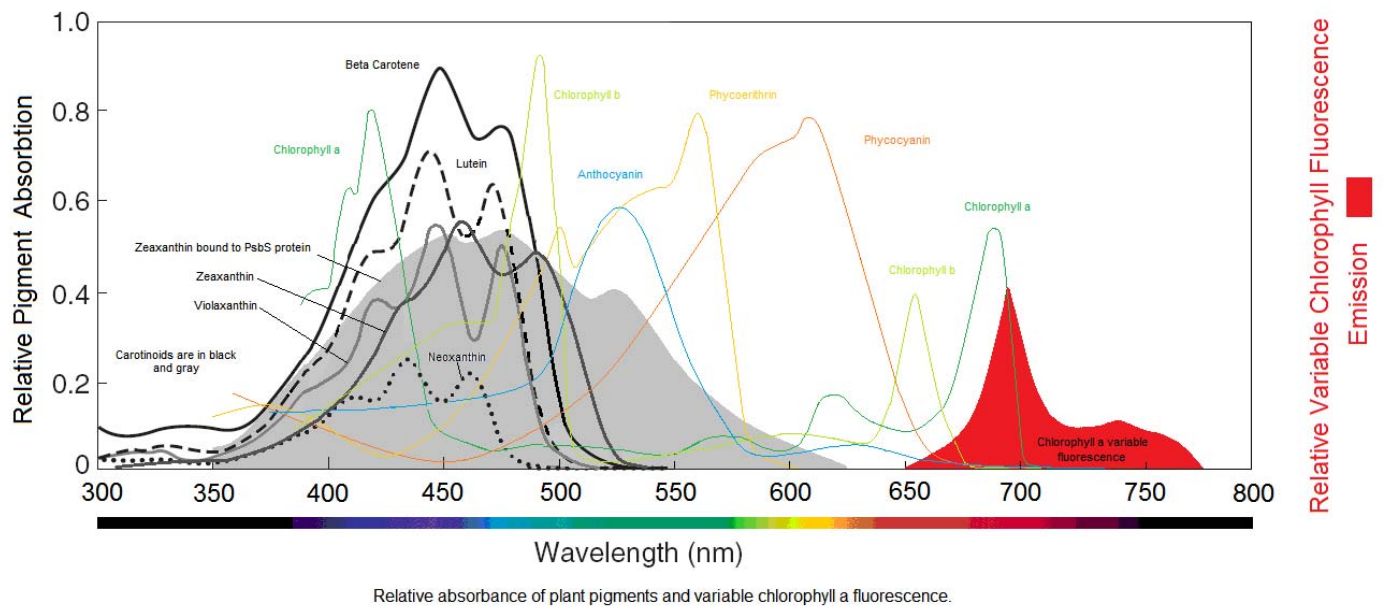
Photoinhibition is a process that can occur at high light levels for extended periods of time. It has been shown that the effects of acute photo-inhibition caused by exposure to high light intensities for an hour or two, can be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter 1998). Where as reversal of chronic photo-inhibition, caused by several hours of exposure, starts to relax or repair at about 40 minutes and may take 30 to 60 hours to fully relax or repair under dark adaptation (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998). When making longer quenching and quenching relaxation parameter measurements related to photo-inhibition and photo-damage mechanisms that are common in chronic high light stress, high heat stress, cold stress and over wintering stress, one should understand that it can take days for full relaxation or repair of the non-photochemical quenching parameters, q_I , to pre-stress conditions. To get an accurate control value for F_m and F_o under chronic photo-inhibition conditions (components of non-photochemical quenching parameters) it is common to dark-adapt for a full night, or 24 hours. (Maxwell and Johnson 2000). 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 value in samples that do not have the same F_v/F_M because F_v/F_M 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.

Other plant pigments

Carotenoids are present in the antenna systems and reaction cores and in other locations. They include alpha and beta carotene, and xanthophylls: lutein, zeaxanthin, violaxanthin, antheraxanthin and neoxanthin. 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 in plant photo-protection to dissipate excess light energy, the blocking of free oxygen and organic radical, and 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 violaxanthin converts to zeaxanthin as light levels increase, and that zeaxanthin binds with the protein PcbS in a photoprotective role that shifts the absorption spectrum (Aspinall-O'Dea 2002). 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 sunflecks, 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).



Relative absorption of Zeaxanthin, and Zeaxanthin bound to PsbS protein are adapted from Aspinal-O'Lea M. (2002). Chlorophyll a, & b absorption spectra, chlorophyll a emission spectra, anthocyanin absorption spectra are adapted from Papageorgiou & Govinjee (2004). Relative absorption of Lutein, Beta Carotene, Neoxanthin, and Violaxanthin adapted from Lichtenthaler 2001

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 specific types of plant stress measurement (Desk Top Plant Stress Guide). Some protocols will work for usable early detection of some types of plant stress, and on other types of plant stress, that same protocol may not work until the plant stress type is severe. The most used protocols, F_v/F_m , 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, state transitions, photoinhibition, 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 site of carboxylation or C_c . 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.

F_V/F_M protocol

F_V/F_M 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 F_V/F_M are lower than instruments that measure multiple measuring protocols, F_V/F_M is the most used chlorophyll fluorescence measuring parameter in the world. F_V/F_M 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).

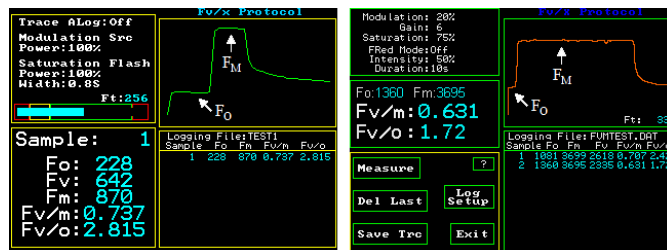
$$F_V/F_M = (F_M - F_O) / F_M$$

F_M 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, F_M is the highest fluorescence value or an average of the highest values.

F_O is minimum fluorescence. It represents fluorescence emitted from antennae before the any Q_A (quinone A) has been chemically reduced in the leaf. Using a modulated light fluorometer, a very weak modulated light, $< 1 \mu\text{mol}$, 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. F_O is then pre-photosynthetic fluorescence before any Q_A has been reduced (Zhu 2005). In continuous fluorometers, F_O 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_B^- non-reducing centers increase, raising F_O , and reducing F_V/F_M . F_M can also be reduced by larger plastoquinone pools and higher plastoquinone reoxidation constants (Zhu 2005). The maximum F_V/F_M value for a healthy plant under ideal conditions is between 0.79 and 0.83. for most species. Lower values indicate plant stress. While F_V/F_M 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)

F_V/F_M 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 F_V/F_M 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.

Y(II) protocol

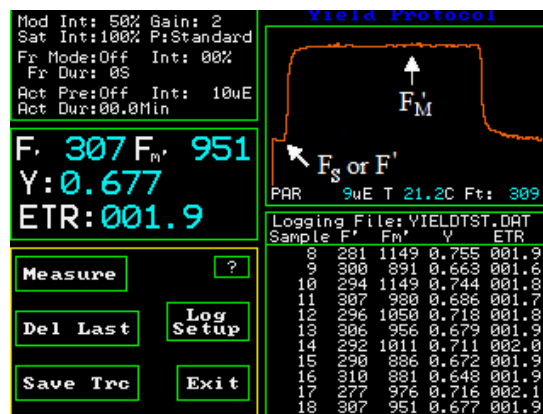
Y(II) also known as $\Delta F/F_M'$, and Φ_{PSII} $Y(II) = (F_M' - F_S) / F_M'$

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. It takes between 15 to 20 minutes at a fixed light level to reach steady state photosynthesis (Maxwell and Johnson 2000).

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 F_V/F_M . For example F_V/F_M 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)

Y(II) works very well for most types of plant stress measurement in C_4 plants, including drought stress. In C_3 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).



This is a graphic display of the OS1p using the Y(II) measuring protocol.

rETR or J- Electron Transport Rate

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.

$rETR = 0.84 \times 0.5 \times PAR$ is a relative equation using average values.

or $rETR =$ the leaf absorbance x decimal fraction of PSII reaction centers x PAR (Photosynthetically Active Radiation).

Leaf absorbance can vary for 0.70 to 0.90 . (Eichelman H. 2004), and the ratio of PSII 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, Edwards and Laisk are good sources for information on individual species and conditions. The ratio of PSII reaction centers to PSI reaction centers is different for leaves grown in shade than grown in the sun (Anderson 1995) and under carbon deficiency (Zell 2010), (Anderson 1999) (Elgass 2011).

It has been stated that ETR or J should only be used for comparisons on leaves where the leaf absorbance and ratio of PSII to PSI reaction centers have been measured (Baker 2008). The ratio of red light absorbance to blue light absorbance changes with light intensity (Bernacchi 2002). 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, however, a new method uses spectral analysis in vivo at room temperature (Elgass 2011).

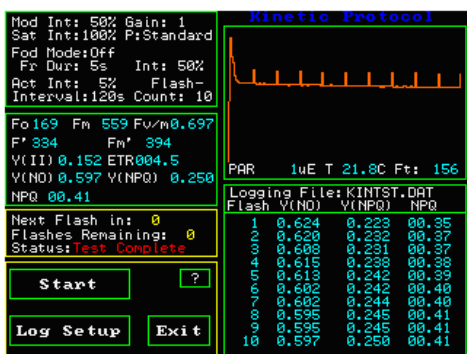
Quenching measurement protocols

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.

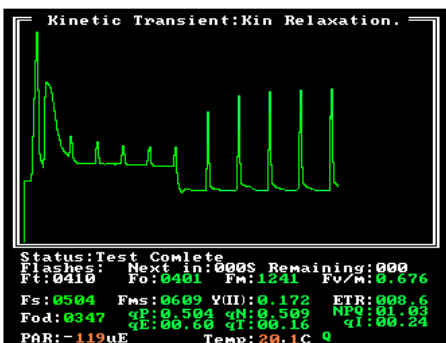
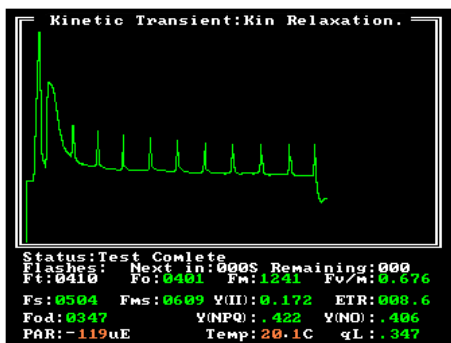
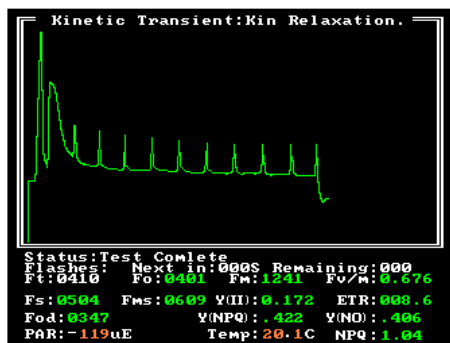
Photochemical quenching is a measure of the fraction of still open PSII reaction centers.

Non-photochemical quenching is a measure of heat dissipation impacted by photo-protection mechanisms, 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).



The display on the left shows the OS1p using the Hendrickson lake model quenching protocol with NPQ resurrected from the puddle 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 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.

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. qP , 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.

Others have also come up with more simplified equations that eliminate the need for the measurement of F_o' and approximate the measurements made by Kramer. F_o' 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 F_o' (or F_{od}) 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 Hendrickson's equations provide lake model parameters without the use of the controversial parameter F_o' . Today, all of these methods are still in use.

Kramer's lake model equations:

$$Y(II) = (F_M' - F_s)/F_M' \text{ or } \Delta F_M' / F_M'$$

$$Y(NO) = 1/(NPQ+1+qL (F_M/F_o - 1))$$

Hendrickson's lake model equations:

$$Y(II) = (F_M' - F_s)/F_M' \text{ or } \Delta F_M' / F_M'$$

$$Y(NO) = F_s/F_M \text{ or } F'/F_M$$

$$Y(NPQ) = (F_s/F_M') - Y(NO) \text{ or } (F/F_M') - Y(NO)$$

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

$$NPQ = Y(NPQ)/Y(NO) \text{ or } NPQ = (F_M - F_M')/F_M'$$

Puddle model equations:

$q_P = (F_M' - F_s)/(F_M' - F_0)$ or $q_P = F_M' - F'/(F_M' - F_0)$ Above 0.4, F_0' should replace F_0

$q_N = 1 - ((F_M' - F_0) / (F_M - F_0))$ Above 0.4, F_0' or F_0' should replace F_0

$NPQ = (F_M - F_M')/F_M'$ or $NPQ = (F_M - F_M')/F_M'$

$NPQ = q_E + q_T + q_I$

$q_E = ((F_{ME} - F_M')/(F_M - F_M'))$ 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 q_E . Times of up to ten minutes have been reported.

$q_T = ((F_{MT} - F_{ME}) / (F_M - F_M'))$ F_{MT} is the relaxation saturation value at twenty minutes in the dark.

$q_I = ((F_M - F_{MT}) / F_M - F_M')$ Relaxation of q_I starts at about forty minutes and can take up to sixty hours. q_I can be determined from the dark adapted F_M measurement and the saturation pulse after 20 minutes used for q_T

Since NPQ has been resurrected for the lake model, the quenching parameters q_E , q_T , and q_I are also valid in the lake model.

Measurement of q_E , q_T and q_I

q_E , q_T and q_I 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 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 minutes while q_E , and q_T relax. 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_0' . The advantage of NPQ over q_N 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 q_N values lower than 0.6. The range of NPQ is affected by ΔpH of the thylakoid lumen which is an important aspect of photosynthetic regulation, 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).

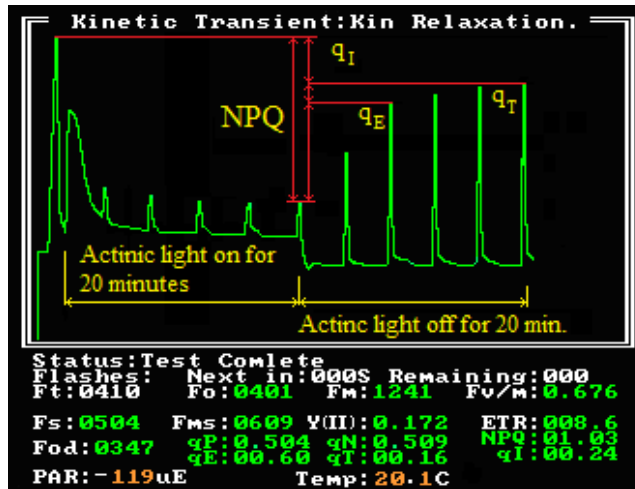
q_N (puddle model quenching protocol parameter) is similar to NPQ but requires F_{od} or $F_{o'}$ in the calculation. q_N is defined as the coefficient of non-photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_v) and not the minimal fluorescence (F_o). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, $F_{o'}$ (or F_{od}) should replace F_o in q_P equations. q_N 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)

q_P (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. q_P is defined as the coefficients of photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_v) and not the minimal fluorescence (F_o). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, $F_{o'}$ (or F_{od}) should replace F_o in q_P 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 F_{od} 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 F_{od} 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)

q_E (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 q_N into q_E , q_T , and q_I instead of NPQ (Lichtenthaler 1999) The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008).

q_T (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).

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).



This is an OS5p quenching relaxation protocol graph with captions. All of these parameters except F_v/F_m and its components are, by definition, only valid at steady state photosynthesis. Because it takes state transitions between fifteen to twenty minutes to adjust in land plants, a stable actinic light source is used to radiate light on a leaf for twenty minutes. At this point, the actinic light is turned off and saturation pulses are used in the dark to show the relaxation of the various plant mechanisms. Saturation pulses must be at least one to two minutes apart to prevent saturation pulse NPQ (Roseqvist and van Kooten 2006). Acute photoinhibition takes up to 30 min to relax or repair, and it can take up to sixty hours for chronic photoinhibition to relax or repair. Notice that F_m is the reference for these values. For that reason, one should never compare nonphotochemical quenching measurements on samples that have a different F_v/F_m . Since NPQ has been resurrected to the lake model in the Hendrickson equations, q_E , q_T , and q_I can also be used with lake model. q_E and q_T overlap to some degree (Muller 2001).

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 Kii rats & 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 Kii rats & 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).

q_L (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 Kii rats & Gerald E. Edwards 2004).

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

Hendrickson lake model quenching parameters

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Y(II) = (lake model) quantum yield of photosynthetic energy. The equation is the same as for Y, or $\Delta F/F_m' \cdot (F_m' - F_s) / F_m'$ 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_o' . 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 ΔpH of the thylakoid lumen, and the xanthophyll cycle which are an important aspect of photosynthetic regulation. 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

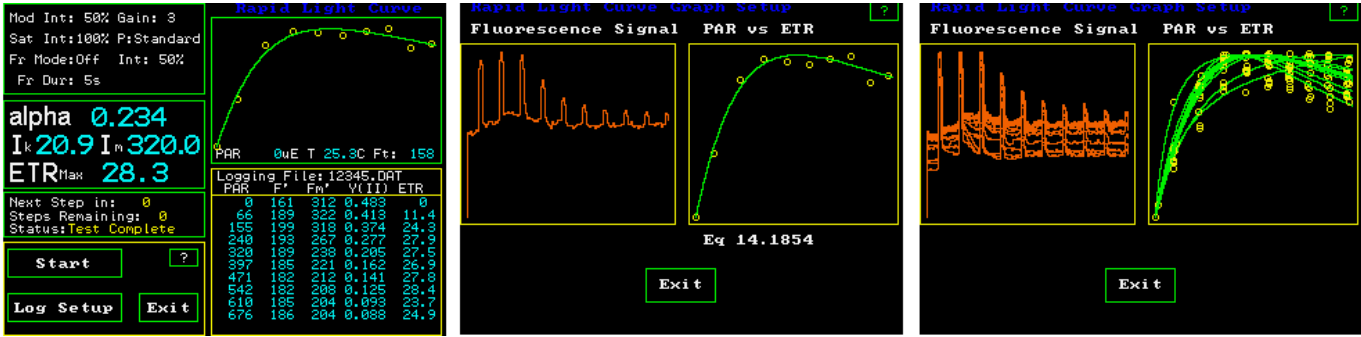
Light Curves. – These are protocols that step through different light levels to find the Y(II), rETR, and other values at different PAR levels. Measurement is only made after the plant has reached steady state photosynthesis. They can start low and move higher or start high and go lower. It takes between 15 minutes to 20 minutes at a given light level to reach steady state photosynthesis (Maxwell and Johnson 2000).

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 (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 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).



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

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, $rETR_{MAX}$ - relative maximum electron transport rate, α is the initial slope of the line at low PAR values, I_K is the point of minimum saturation level = $rETR_{MAX} / \alpha$, and I_M is the $rETR_{MAX}$ PAR level. It is common to use curve fitting software to graph the protocol and determine the values. (Application note on RLC)

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.

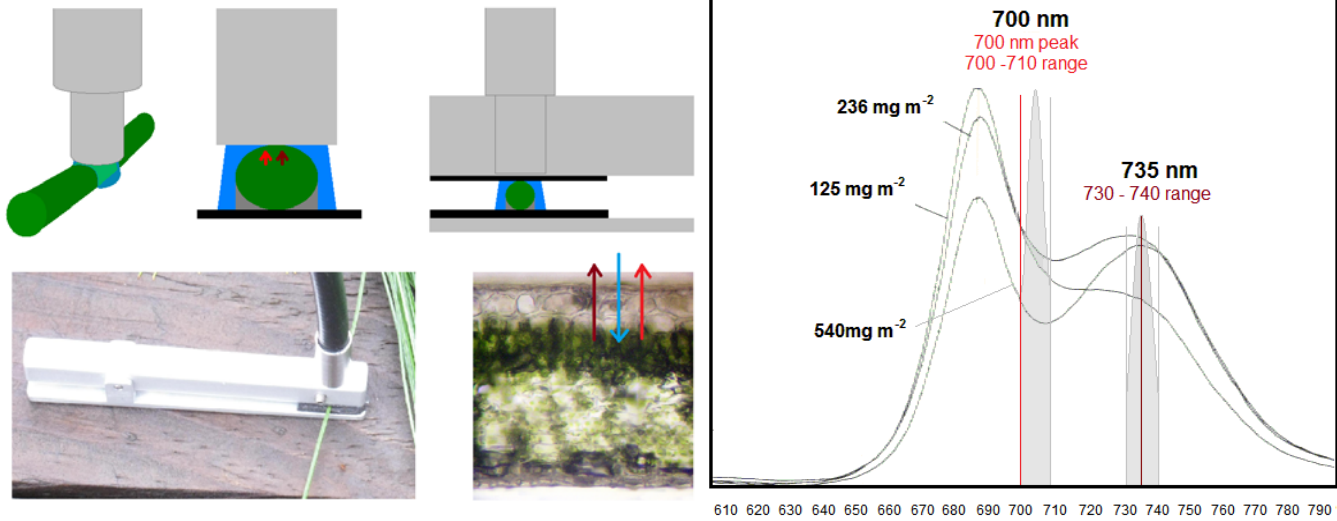
According to Neil Baker, chlorophyll fluorescence is not normally capable of measuring nitrogen plant stress until it is severe, and sulfur plant stress until starvation levels have been reached. (Baker 2004) Several research groups have worked on various fluorescent ratios in an attempt to measure chlorophyll 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, Sampson 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, 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 excited 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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