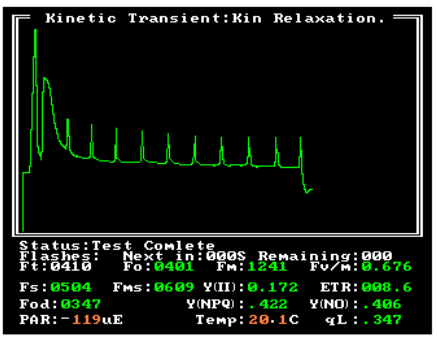
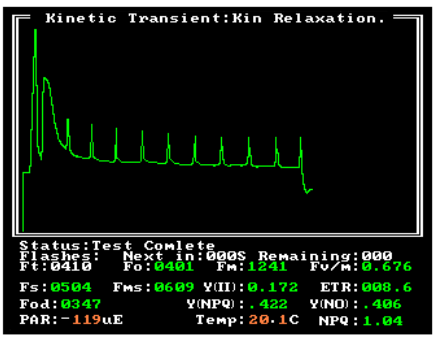
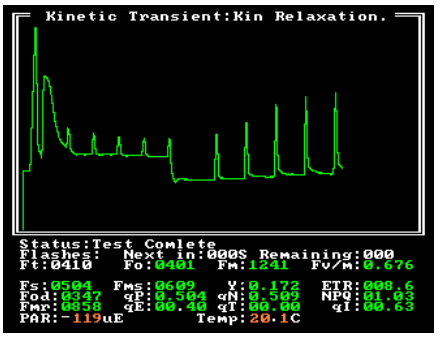
Quenching Measurements using Chlorophyll Fluorescence, Understanding the Light - Dark Kinetic Trace - 2014



Kramer 2004 lake model parameters



Hendrickson 2004 lake model, with NPQ resurrected from the puddle model by Klughammer 2008



Quenching relaxation for the lake and puddle model

Introduction

This article is an overview of the value and limitations of quenching measurements and provides an understanding of photochemical and non-photochemical quenching measurements. The puddle model, the Kramer lake model, and the Hendrickson lake model with NPQ resurrected from the puddle model by Klughammer & Schrieber will be reviewed, and practical considerations are added to the discussion.

Furthermore, quenching relaxation measurements will be discussed including the kinetic traces used in measuring photoprotective mechanisms q_E, state transitions q_T, chloroplast migration q_M, a proposed longer acting xanthophyll mechanism called q_Z, and photoinhibition q_I.

These measurements are usually determined after overnight dark adaptation and the after illumination a specific light level until steady state photosynthesis has been reached. However, Klughammer (2008) states that Y(NO) is not limited to steady state measurement if his simplified equations are used. Without a good understanding of the mechanisms that affect the light trace, machine artifacts and user errors can be included in measurements.

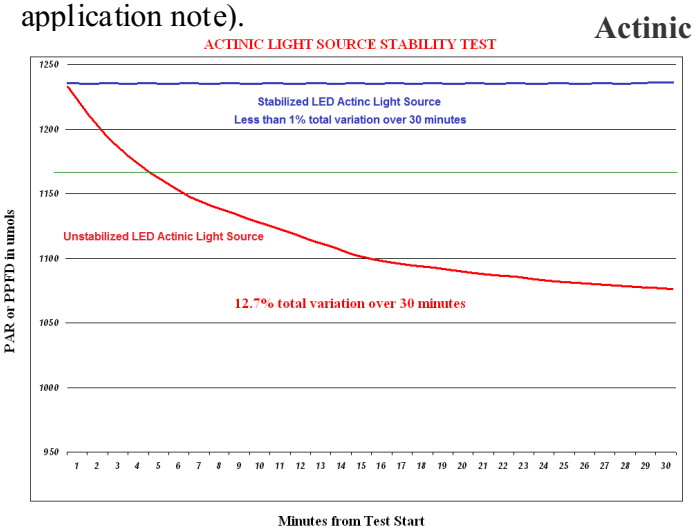
The chlorophyll fluorescence signal

Variable chlorophyll fluorescence of photosystem II has been found to provide significant information regarding light reaction of the photosynthetic processes, plant health, and plant stress measurement. In photosystem II, light energy is absorbed and used in photochemistry, photo-protective regulated heat dissipation, unregulated passive heat dissipation, or given off as variable chlorophyll fluorescence. This process is competitive and conditions that optimize photochemistry decrease heat dissipation and chlorophyll fluorescence. There is a linear relationship between the quantum yield of PSII or Y(II) measurements made with chlorophyll fluorescence and carbon assimilation in C_4 plants. In C_3 plants, the relationship is curvilinear and in C_3 plants, this relationship can be delayed by photorespiratory conditions. While PSI fluorescence exists, it is low, and does *not* vary in intensity. As a result variable chlorophyll fluorescence of PSII if of most importance and has also been found to be sensitive to most types of plant stress (Baker 2008).

Proper dark adaptation is very important for quenching measurements

The dark-light kinetic curve used in quenching measurements begins with dark adaption of the sample leaf. It is common when making quenching measurements to dark-adapt *for a full night*, or even 24 hours (Maxwell and Johnson 2000). In some cases, longer times may be appropriate. Lichtenthaler (2004) found that it can take up to 60 hours for complete relaxation of NPQ in plants subjected to chronic photoinhibition high light conditions for several hours. *All non photochemical quenching measurements use F_M found in F_V/F_M as a reference* and some parameters also use F_O (Schreiber 2004). For this reason it is important to ensure that one starts with a properly dark adapted sample because all measurements are made relative to these values. As a result, one should never compare samples that have a different F_V/F_M values (Baker 2008). In the field, pre-dawn quenching measurements can provide a way to measure plant stress; however, it is important to understand that these values may be still be affected by recent light history (Maxwell and Johnson 2000) (Lichtenthaler 2004). Leaves with dissimilar light histories, or different F_V/F_M values, or of different species should not be compared using non-photochemical quenching parameters. (Maxwell & Johnson 2000). Baker (2008) states that only leaves with the same F_V/F_M values should be compared. (For more details on dark adaptation please request the Opti-Sciences dark adaptation application note).

Actinic Light Source



Intensity curve of a stable white actinic light source over a 30 minute time period

Compared to white light LED that is not stable.

The light intensity decline shown by the non stable white light LED is common. This issue is also found in Red Light LEDs (data unpublished).

Actinic Light Source

Most built-in fluorometer light sources used as actinic sources for quenching measurements and light curves decline in intensity during these measurements if the intensity is not stabilized. This is due to the fact that heat from the internal light source reduces light output. It can happen to halogen light sources and to LED light sources. When this happens, the photosynthetic sample may never really reach steady state photosynthesis, a process that takes between fifteen and twenty minutes (Maxwell and Johnson 2000) at lower and medium light levels and can take from twenty minutes to thirty five minutes at near saturating light levels due to chloroplast migration (Cazzaniga S. 2013). Such actinic light sources can produce errors in all quenching and quantum yield of PSII values. Opti-Sciences provides a stable LED actinic light source with the OS5p+ and the OS1p. To achieve actinic light intensity stability, it is recommended that the PAR clip is used with a tripod and a dark shroud. The PAR sensor measures the fluorometer actinic light output at the leaf and adjusts the intensity of the actinic light source every minute automatically, maintaining a stable light intensity. *Furthermore, recent research has found that actinic light sources must be white or have an intense blue spectrum for chloroplast migration to occur as it does in nature (Cazzaniga 2013). See the section on q_M for more information.*

Multi-flash - **based on Loriaux (2006), & Loriaux, (2013)**

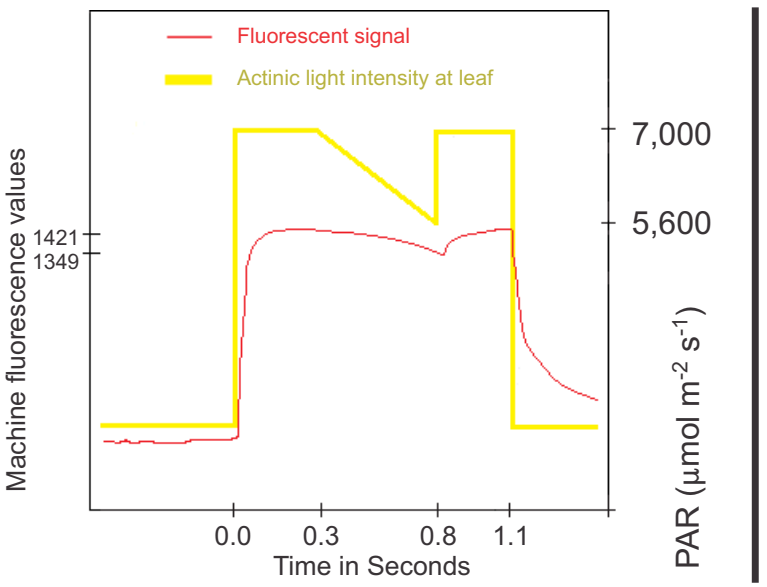
Saturation pulses used with modulated fluorometers are designed to close all PSII reaction centers.
The maximum fluorescence intensity value, of the saturation flash, F_M', is used in most measurements including, quantum yield of PSII Φ_{PSII} (also called Y(II) or $\Delta F / F_M$'), *J* (or ETR), and in all quenching protocol parameters.

While 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 Q_A is prevented by fast turnover of the plastoquinone pools. (Margraph 1990, Loriaux 2013). With this in mind, Y(II) and ETR measurements taken under these conditions, can be underestimated. In a poster, researchers that included Bernard Genty, the developer of quantum yield of PSII, verified the issue, and developed a method for F_M' correction. 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 (Photosynthetically Active Radiation), to determine the F_M' fluorescence level using an infinitely intense saturation pulse, without causing damage to the plant and without closing all of the reaction centers.

Studies by Earl (2004), and Loriaux (2006), have compared chlorophyll fluorescence measurement results with gas exchange measurements and found that by using multiple saturation flashes, and regression analysis, an infinite fluorescent saturation light flash intensity can be determined and used to correct Φ_{PSII} or (Y(II)) and *J* (ETR) measurements. *Research has shown that Y(II) measurements, taken under high actinic light conditions, can be underestimated with up to a 22% error, and there can be up to a 41% error in ETR values if this method is not used.*

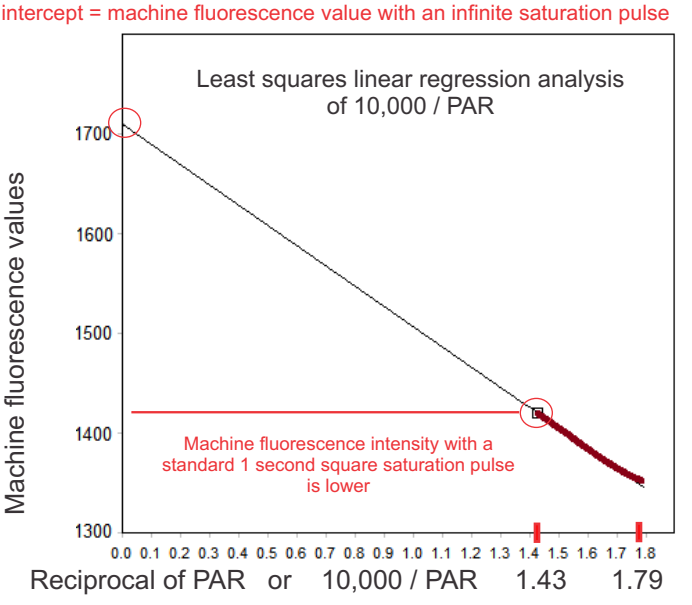
This standard option is provided on the OS5p+, the i*FL*, and OS1p instruments. It is *available for all Light adapted and quenching protocols,* and it can be turned off or on. The method described by the Loriaux, Burns, Welles, McDermitt, & Genty (2006) and expanded by Loriaux, Avenson, Welles, McDermitt, Eckles, Riensche, & Genty (2013), provides the most accepted method currently available. According to the science, the OS5p+ provides the optimal saturation intensity of 7,000 µmols, optimal light ramping of 20%, and a ramping rate less than 0.01 mol $m^{-2}s^{-2}$. While some adjustment is possible, the default protocol has been optimized for most applications.

Representation of how the Multiple Phased Flash works



Least squares linear regression of 10,000 / PAR values

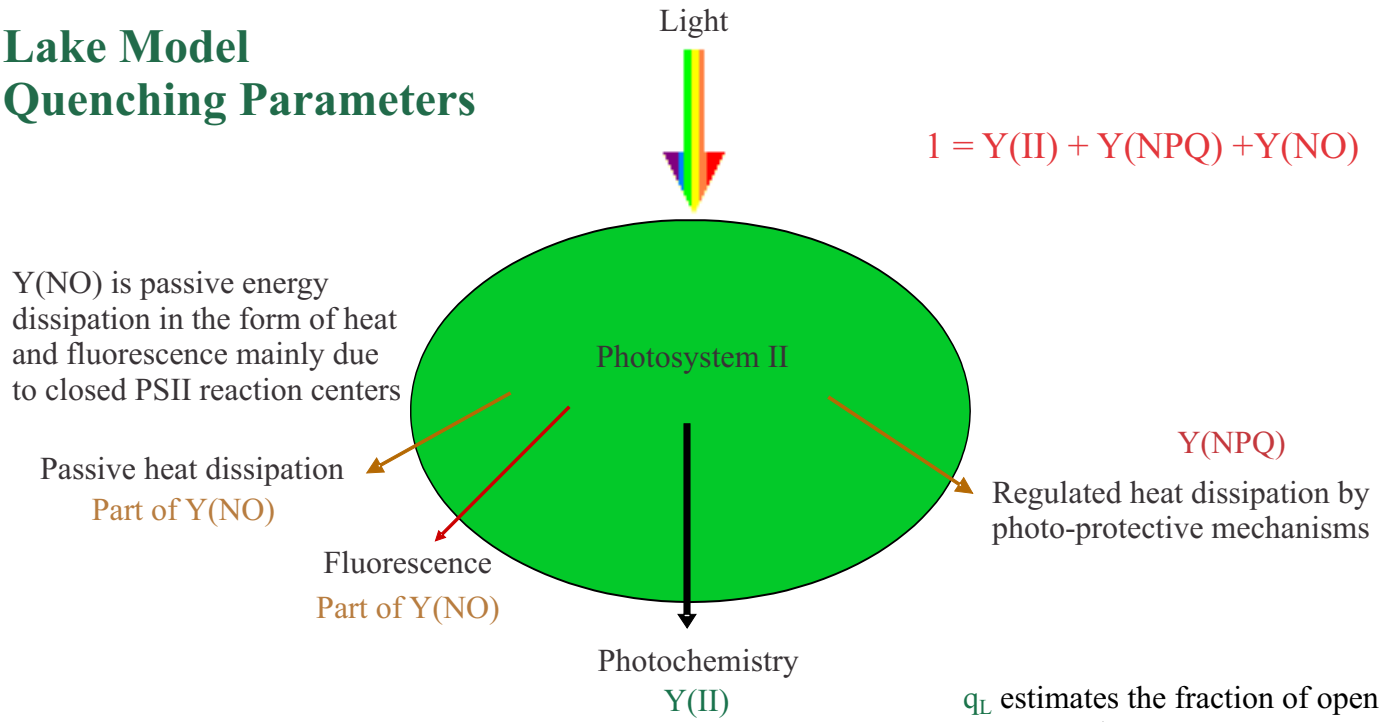
y intercept = machine fluorescence value with an infinite saturation pulse



The first saturation flash step, shown on the left, 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 ramping rate is less than 0.01 mol photons $m^{-2}s^{-2}$. The final phase is at 7,000 µmols to check for saturation pulse NPQ. Recent studies have shown that those setting provided optimal results for plants that have been tested. (Loriaux 2013). A rolling 25ms eight point average is used to determine maximum F_M'

The graph on the right represents the Loriaux, (2006) & Loriaux (2013) method for estimating Fm' with an infinitely intense saturation flash. Least squares linear regression analysis of the reciprocal of PAR (or 10,000 / PAR) allow determination of the y intercept, which represents the machine fluorescence value with an infinite saturation flash.

Lake Model
Quenching Parameters



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.

Another researcher, Luke Hendrickson (2004), developed a more simplified set of equations for the lake model that eliminate the need for the measurement of Fo and Fo' and approximate the measurements made by Kramer.

Hendrickson's (2004) work offered such a 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 Fod) measurements. Furthermore, Hendrickson does not provide a parameter like q_L to estimate the fraction of open PSII centers.

From Hendrickson's work, and earlier works by Cailly (1996) and Genty (1989, 1996), Klughammer and Schreiber derive simplified equations that allow for Hendrickson's parameters, and also allow users to resurrect the popular NPQ parameter and reconcile NPQ measurements with the lake model.

Opti-Sciences offers the user the ability to choose which parameter set they want to use. One can choose Kramer parameters, Hendrickson - Klughammer & Schreiber parameters or puddle model parameters. The use of puddle model parameters have been retained because there is a significant volume of work done using the older puddle model parameters that may be valuable for comparison. In addition, the reconciliations of NPQ with lake model by Klughammer and Schreiber leave open the opportunity for separation of q_E, q_M, q_Z, q_T and q_I, quenching relaxation parameters, in lake model work.

Lake Model, Puddle Model, and Quenching Relaxation Equations

Kramer, and Hendrickson's lake model parameters account for all light that is absorbed by PSII. All parties agree with the following equation.

Kramer's equation is $1 = Y(II)+Y(NPQ)+Y(NO)$

Y(II) is quantum yield of photochemical energy in photosystem II, also known as ΔF/Fm' or (Fm'- Fs')/ Fm'

For comparison purposes, the differences in the equations are listed below.

Lake model parameters

Kramer's equations

$Y(II) = (F_M' - F_S)/F_M'$ or $\Delta F/ F_M'$

$qL = ((F_M'-F_S)/ (F_M'-F_O))(F_O'/F_S)$ or $qL = qP(F_O'/F_S)$

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

$Y(NPQ) = 1- Y(II)-Y(NO)$

Hendrickson's equations with NPQ resurrected to the lake model by Klughammer and Schreiber's equation

$Y(II) = (F_M' - F_S)/F_M'$ or $\Delta F/ F_M'$

$Y(NO) = F_S'/F_M$ or F'/F_M

$Y(NPQ) =(F_M/F_M') -Y(NO)$

$NPQ= Y(NPQ)/Y(NO)$ or $NPQ = (F_M-F_M')/F_M'$

Puddle model parameters

$q_P = (F_M' - F_S)/(F_M' - F_O)$ Above q_P values of 0.4, F_O' replaces F_O

$q_N = 1- ((F_M'- F_O) / (F_M - FO))$ Above q_N values of 0.4, F_O' replaces F_O

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

Quenching relaxation parameters

$NPQ=q_E+q_M+q_I$ or $NPQ=q_E+q_Z+q_I$ or $NPQ=q_E+q_T+q_I$

$q_E = ((F_{ME} - F_M')/(F_M-F_M'))$ F_{ME} is the relaxation saturation value at several seconds to about 7 minutes in the dark. q_E is a measure of fast acting photoprotective plant mechanisms.

$q_M =((F_{MM} - F_{ME}) / (F_M - F_M'))$ F_{MM} is the relaxation saturation point at about 20 minutes to 35 minutes. q_M is a measure of a longer term photo avoidance mechanism chloroplast migration.

$q_Z =((F_{MZ} - F_{ME}) / (F_M - F_M'))$ F_{MZ} is the relaxation saturation point at about 15 minutes to 30 minutes. q_Z is a term used for an unknown longer term xanthophyll photoprotection likely to be q_M.

$q_T = ((F_{MT} - F_{ME}) / (F_M-F_M'))$ F_{MT} is the relaxation saturation value at 15 to 20 minutes in the dark at lower and medium light levels. q_T is a measure of state transitions where they exist at lower light level.

$q_I = ((F_M -F_{MT}) / F_M - F_M'))$ 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 Fm measurement and the saturation pulse after 30 to 35 minutes used for q_T. q_I is a measure of photoinhibition.

Puddle model event times for q_E, q_T and q_I were taken from Lichtenthaler (1999). Descriptions of q_E, q_T and q_I are taken from Muller P., Xiao-Ping L., Niyogi K. (2001) and Lichtenthaler (1999). Nilkens (2010) was used for q_Z event times and descriptions. Cazzaniga (2013) was used for q_M event times and descriptions. In field plants , the relaxation time for q_E, q_T, and q_I can be extended (Baker 2008).

Definitions

Actinic light is the light incident on the photosynthetic sample that provides energy to drive the photosynthesis process. This light may be either sunlight or artificial light.

Far red light is a light source that provides light at 730 nm to drive PSI without driving PSII. This has the effect of draining the remaining electrons from PSII quickly to provide a completely oxidized state in PSII. It is used for determination of F_O'.

Modulated light source This is the light source that makes light adapted quantum photosynthetic yield measurements possible along with direct measurements of F_O and F_O'. The modulated light source is used at an intensity range that is too low to allow reduction of Q_A, and yet allows fluorescence measurement of pre- Q_A reduction of F_O, and post light adapted F_O'. This light source is turned off and on at a particular frequency. The frequency is adjusted either manually or automatically for optimal application usage. Opti-Sciences adjusts the frequency automatically for specific measurement conditions.

Saturation pulse is a short pulse of intense light designed to fully chemically reduce all available photosystem II reaction centers. For higher plants, the optimal duration of the saturation pulse is between 0.5 seconds and 1.5 seconds (Rosenqvist and van Kooten 2006). In Algae this time is between 25 milliseconds to 50 milliseconds. Various color light sources have been used as saturation light sources including red light and white light sources. The intensity is designed to be high enough to chemically reduce or to close all PSII reaction centers. It may be a halogen light source or and LED light source. Opti-Sciences allows the saturation pulse duration to be set from 0.1 seconds to 1.5 seconds. However, Opti-Sciences ensures correct saturation pulse duration by using a rolling 25 millisecond eight point average to determine F_M and F_M'. As a result, correct measurements will be made on all samples as long as the saturation duration is long enough. Saturation intensities in the range of 1,500 umols are high enough to saturate sun grown leaves, while intensities of a few hundred µmols are usually high enough to saturate shade leaves or plants grown indoors. Currently, the OS30p+ offers saturation intensities up to 6,000 µmols, the OS1p and the OS5P+ offer saturation intensities up to 11,000 µmols. For a review of light adapted test issues, review the section on Multiflash.

F_O is the dark adapted initial fluorescence measured with a modulated light intensity too low to photochemically reduce Q_A.

F_M is maximal fluorescence measured during the first saturation pulse after dark adaption. All available reaction centers are fully reduced and closed. F_M represents multiple turnovers of Q_A. The rise ends with maximum chemical concentrations of $Q_A^- Q_B^{2-}$ and PQH_2. It ends with the reoxidation of PQH_2 by the cytochrome b_6f complex.

F_S also known as F' is the fluorescence level created by the actinic light at steady state conditions. Initially the value of F' is high and then decreases over time to steady state values due to the initiation of electron transport, carboxilation, and non-photochemical quenching and either q_M, q_Z, or q_T.

F_M', also known as F_{MS}, is a saturation pulse value that is not dark-adapted. It is equivalent to Fm in a light adapted state. They are lowered values due to NPQ (non-photochemical quenching). When measuring sample has reached steady state in photosynthesis, it is used to calculate quantum photosynthetic yield along with Fs. Fms at steady state is also used to calculate q_N, NPQ, q_P, q_L, Y(NPQ), q_E, q_M, q_Z, q_T, and q_I. Klughammer claims that Y(NO) does not require steady state photosynthesis.

F_O', also known as **F_{od}** , is the minimal fluorescence value after the actinic light has been turned off and after the far red light is turned on for several seconds. It represents **F_O** with non-photochemical quenching. It may also be described as minimum chlorophyll fluorescence yield with maximal opening of all PS II reaction center traps in a light-acclimated state.

F_t is the instantaneous fluorescent signal received by the fluorometer.

$F_V/F_M = (F_M - F_O)/F_M$ Dark adapted test - a normalized measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open. 0.79 - 0.83 are the approximate optimal values for most plant species with lowered values indicating plant stress. (Maxwell K., Johnson G. N. 2000) (Kitajima and Butler, 1975). Fv/Fm has a photochemical component and a non-photochemical component (Baker 2004). *F_V/F_M is a fast test that usually takes less than two seconds but may take between 6 seconds and 12 seconds if the sample is pre-illuminated with far red light.*. It is important to properly dark adapt samples for this test. F_O will be raised and F_M will be lowered if dark adaption is inadequate. Since dark adaption requirement can vary with light history, species, varieties, mutants, and sun vs. shade leaves, testing should be done to ensure proper dark adaption. (Kitajima and Butler, 1975) (For more information on dark adaption there is an application note available from Opti-Sciences.)

Y= Y(II) or FMS – FS / FMS (or ΔF/FM') Effective Quantum Yield of photosystem II- It is a light adapted test – a normalized ration measurement that is an indication of the amount of energy used in photochemistry by PSII under steady-state photosynthetic lighting conditions. (Genty 1989), (Maxwell K., Johnson G. N. 2000), (Rascher 2000) It is affected by closure of reaction centers and heat dissipation caused by non-photochemical quenching. (Schreiber 2004), It is also affected by chloroplast migration at near saturation intensities (Cazzaniga 2013) Y(II) *is a fast test that usually takes less than three seconds.*

Puddle Model Quenching Definitions

NPQ (puddle model parameter and 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' or F_{od}. 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 non-photochemical 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, chloroplast migration (Cazzaniga 2013), 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 parameter) is similar to NPQ but requires F_O' or F_{od} 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, Fo' (or Fod) 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. With application of far red light, 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 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_O' 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)

q_E (puddle model and lake model 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 $\triangle$pH 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 but may be as long as seven minutes in field grown leaves (Baker 2008), (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 and lake model 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). At near higher light levels and near saturation light conditions fluorescence changes starting before qE is ended and lasting for between 20 minutes and 35 minutes has been shown to be the result of chloroplast migration or qM (Cazzaniga 2013).

q_I (puddle model and lake model 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 photo-inhibition 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).

Quenching Relaxation Definitions for Lake and Puddle Model
q_E, q_M, q_Z, q_T, q_I

q_E (puddle model and lake model 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 but may be as long as seven minutes in field grown leaves (Baker 2008), (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).

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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)ph 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 zeazanthin synthesis. Relaxation of q_E is regulate the)ph 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 qZ 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 zeazanthin 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 – chloroplast migration

Cazzaniga S, Osto 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, 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 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 q_T , but extended up to 35 minutes with some 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 q_T and q_Z fluorescence change.

Researchers found that high white actinic light was more effective than high red actinic light at inducing the photoprotective functions of q_M. 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 qE 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 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 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.

Notes for Quenching Relaxation:

The relevance of state transitions measured as q_T fluorescence is highly questionable for a number of photosynthetic organisms including Arabidopsis. The evidence shows that changes previously reported as q_T 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 photon absorption as the source of fluorescence change during light adaptation and during quenching relaxation, in dicot land plants at the very least.

Some prominent researchers see evidence that intermediate component mechanisms that are part of an NPQ measurement, may not be the same in all photosynthetic organisms. There could be a relationship between the phosphorylation found in state transitions and NPQ regulation found in some monocots (corn, barley and rice). There is also some strong evidence that q_T fluorescence, from state transitions, exists in the green algae Chlamydomonas reinhardtii (Depège N., Bellafiore S., Rochaix J-D., 2003). Current research will likely provide some additional surprises in this area, moving forward. 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. 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 Φ_{PSII} , NPQ, g_M, C_C and q_I may all include measuring errors without the a reliable 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 lists15 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 SG., 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.*

Lake Model Definitions

Y(NPQ) is a lake model quenching parameter that 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) is a lake model quenching parameter that 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 Schrieber 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).

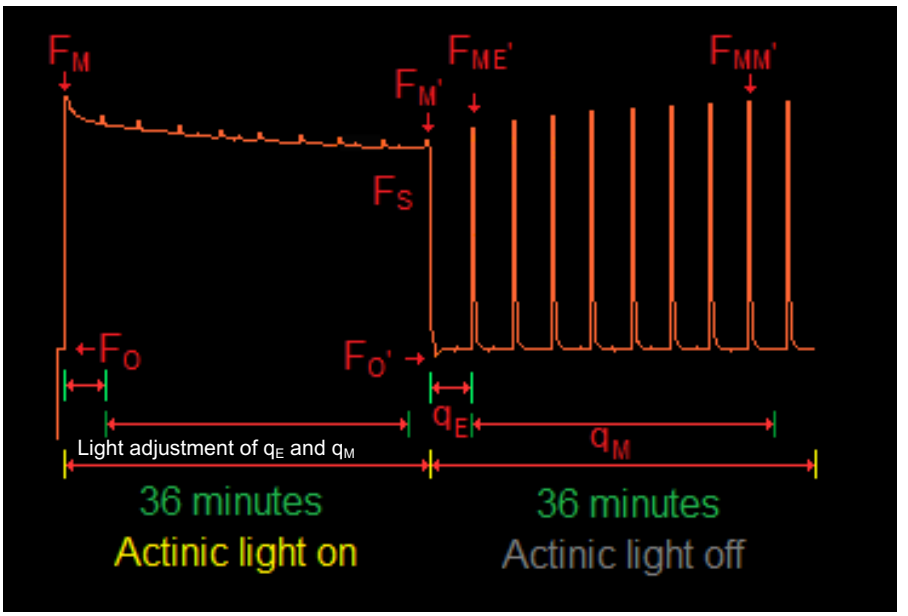
q_L is the lake model quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004)

Y(II) = quantum yield of photosystem II. The equation is the same as for Y, or ΔF/FM'.

$$(F_M' - F_S) / F_M' \quad \text{or} \quad \Delta F / F_M'$$

Puddle model parameter reconciled with the lake model (Klughammer and Schreiber 2008)

NPQ = Y(NPQ)/Y(NO) or NPQ= $(F_M-F_M')/F_M'$ Klughammer and Schreiber reconcile NPQ with the lake model using simplified parameters.



Fluorescence trace with dark adaptation, under high actinic light and then in the dark.

Graph of formula components used in quenching protocols.

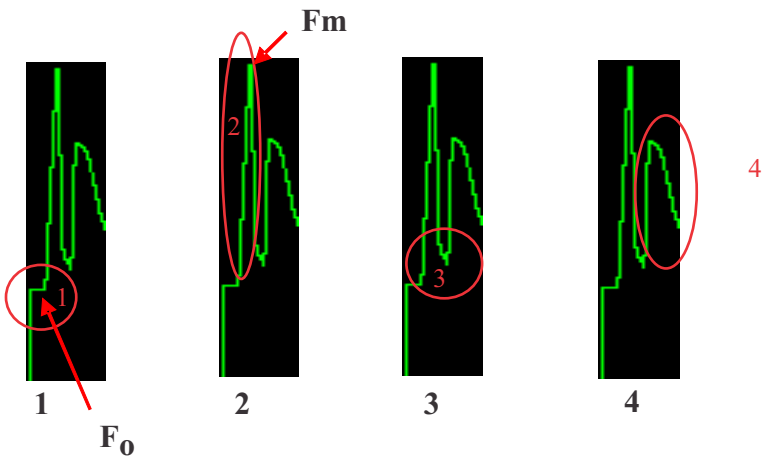
q_E, q_M, q_T, q_Z and q_I may be selected and the times for reporting each may be selected.

F_{MM}' takes 20 to 35 minutes.

F_{MT}' takes between 15 to 20 minutes

F_{MZ}' takes between 20 and 30 minutes

Dissecting the Kinetic Trace



#1 represents a condition that is normally dark adapted (proper dark adaption is required when making non-photochemical quenching measurements) with the sample shielded from any actinic light that would drive photosynthesis. The only light on at this time is the modulated measuring light at about 0.1µmols intensity. This is not enough to drive photosynthesis but it is enough to detect and measure minimum fluorescence from the leaf antennae. In most commercial fluorometers, 30% of the intensity of F_O in C_3 plants is the result of fluorescence from PSI.

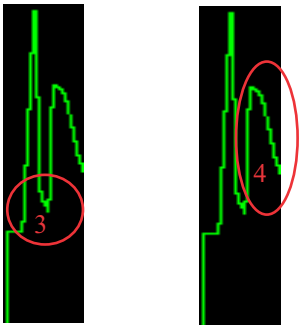
In C_4 plants, 50% of the intensity of F_O is the result of fluorescence from PSI. This contributes to a small error in F_V/F_M measurements and creates an underestimation of maximum quantum efficiency (Baker 2008). PSI fluorescence does not vary in intensity.

#2 shows the first saturation pulse flash. This is a very intense short lasting flash of light that is designed to saturate PSII and close all available reaction centers. For higher plants, the optimal time duration for a saturation pulse has been found to be between 0.5 seconds to 1.5 seconds (Rosenqvist and van Kooten 2005). For Algae and cyanobacteria, the optimal duration of the saturation pulse is shorter, 25 to 50 ms (Schreiber 1995). This allows complete saturation without causing NPQ to underestimate F_M. Saturation pulse durations that are longer than recommended create a rounding of the top back of the saturation pulse caused by a form of NPQ. With dark adapted samples complete closure of all available PSII reaction centers can be accomplished with minimal saturation intensity. PAR Light values of 3,000 µmols or higher are commonly used. Certainly values of 3000 µmols or higher will fully saturate any properly dark adapted sample. Opti-Sciences provides a special algorithm that uses a rolling 25 ms eight point measuring average to determine F_M and F_M'. This ensures that the best value will be measured as long as the saturation pulse duration is long enough. It eliminates saturation pulse NPQ as a source of measuring error.

Most modulated fluorometers offer a wide range of saturation intensities that may be more valuable in closing PSII reaction centers in light adapted conditions. Earl (2004), Markgraf (1990) and Rosenqvist (2006) found that under high light conditions, even the most intense saturation pulse would not completely close all PSII reaction centers available. Earl proposed a method for correction of F_M' values to provide better correlation of ETR (or electron transport rate), with gas exchange measurements. By ramping saturation pulse intensities in a single flash with a total time of 1.0 seconds, the saturation fluorescent intensity at infinity can be determined through linear regression analysis. Loraiux 2013 updated and refined this method. (See the section on Multiflash for more details). While researchers can continue to use the standard square top saturation flash, Opti-Sciences now offers the Loriaux 2013 saturation flash correction for all Y(II) and quenching protocol measurements in the OS5p+ and the OS1p.

The fluorometer sampling time resolution in this type of test is normally lower than in tests used to measure OJIP because one is usually more interested in quenching mechanism measurements over much longer periods of time. For this reason, the rise from F_O to F_M looks more like a sky scraper than a hill with multiple bumps.

The rise from F_O to F_M represents multiple turn overs of Q_A. the rise reaches it's peak at F_M when chemicals $Q_A^-Q_B^{2-}$ and PQH_2 reach maximum concentrations and the cytochrome b6f complex reoxidizes PQH_2 (Zhu 2005, 2012). PSII becomes fully reduced at F_M with all available PSII reaction centers closed.

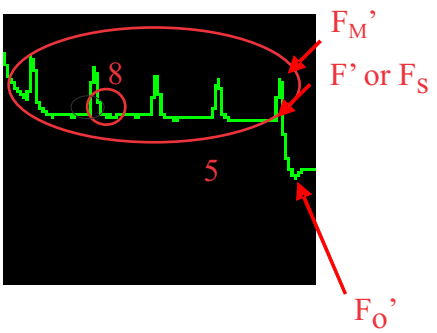


#3

All light sources except the modulated measuring light source are turned off after the saturation flash. The fluorescence graph tails off until it reaches bottom exhibiting non-photochemical quenching related to the saturation flash. During this period Q_A is re-oxidizing but it is a partial condition because there is not enough time for full re-oxidation.

#4

The fluorescence signal rises again due to the turning on of an actinic light source to drive photosynthesis. The fluorescence signal competes for energy with photochemistry and heat dissipation. Since photochemistry and heat dissipation mechanisms have just been initiated, most of the energy goes to fluorescence. The fluorescence signal starts to fall as electron transport and carboxylation begins (Schreiber 2004). Heat dissipation mechanisms are also beginning to affect the signal to drive down the intensity values of both the saturation flashes and the fluorescence signal itself at higher light intensities.

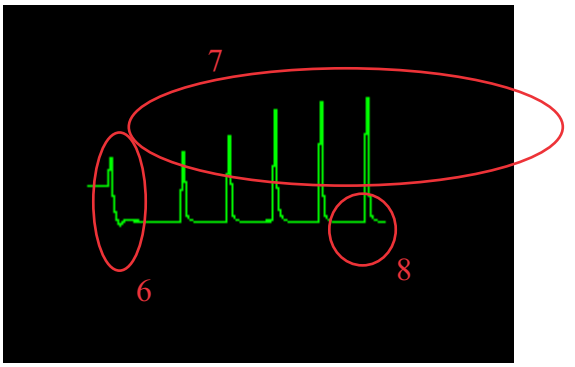


#5

The fluorescence signal continues to drop as full activation of rubisco continues and either chloroplast migration, or state transitions occur. Full activation of rubisco in both algae and higher plants takes between three to four minutes (MacIntyre, Sharkey, Geider 1997). Chloroplast migration, a light avoidance mechanism, takes between twenty to thirty five minutes to adjust. State transitions may be a factor at lower light levels in some plants, and state transitions take between fifteen and twenty minutes (Ruban A.V., Johnson M.P., 2009), (Allen J. F., Mullineaux C.W., 2004), (Lichtenthaler H. K., Burkart S., (1999).

The fluorescence signal and saturation flash intensity are affected by light level, heat and cold, as well as many other forms of plant stress , and the plants ability to deal with plant stress. (see The Opti-Sciences Stress Guide for more information).

The saturation pulse intensity values are driven down by non-photochemical quenching. These are mechanisms involved in photo-protection, light avoidance chloroplast migration, possibly state transitions in some plants, and photoinhibition. The ΔpH of the thylakoid lumen and the xanthophyll cycle adjustments take between two to four minutes to adjust to a new light level (Lichtenthaler 1999), but may take up to seven minutes in field grown plants (Baker 2008). State transitions, where they exist, take between fifteen and twenty minutes to adjusts to a light level, (Ruban A.V., Johnson M.P., 2009), (Allen J. F., Mullineaux C.W., 2004), (Lichtenthaler H. K., Burkart S., 1999). Chloroplast migration at higher light levels near saturation intensities take from twenty to 35 minutes to fully adjust. As a result, steady state photosynthesis takes between fifteen and twenty minutes (Maxwell and Johnson 2000) at lower to medium light intensity levels and from twenty to thirty five minutes at higher light intensities. Under photo inhibitory conditions D1 protien degradation found in PSII reaction centers close PSII reaction centers. Other mechanisms have also been suggested for being involved in photoinhibion.



6

At this point the actinic light source is turned off, and a far red light is turned on for several seconds to activate PSI and drain all remaining electrons from PSII. This results in a quenched measurement of F_O called F_O', the minimum value measured. F_O'is used in the quenching parameters q_P, q_L, q_N, and Kramer's Y(NPQ), and Y(NO). F_O' has also been used in q_E, q_T, and q_I when q_N is used in place of NPQ. After five to ten seconds, the far red light is turned off.

The Far red light at 735 nm is too long to drive PSII, but it will drive PSI. As a result PSII becomes reoxidized.

7

This section of the graph is used for non-photochemical quenching relaxation measurements required in the puddle and the lake model for separation of q_E, q_M, q_Z, q_T, and q_I. During this phase of the graph, the actinic light is automatically turned off and the sample is in the dark. Only the modulated light and saturation pulses are used here. The increase in the peak height is a result of the relaxation of non- photochemical mechanism including ; photo-protective mechanisms, chloroplast migration, possibly state transitions for some plants, and eventually photoinhibition. Lichtenthaler found that the relaxation of photo protective mechanisms that involve Δph of the thylakoid lumen and the xanthophyll cycle takes between two and four minutes. However Baker (2008) found that this time can be extended to seven minutes in field grown plants. A saturation pulse at the end of this period can be used to measure q_E (puddle model or lake model) photoprotective mechanisms Lichtenthaler (1999). The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008).

Where state transitions have been found to exist, the relaxation of state transitions takes between fifteen and twenty minutes, so a saturation pulse after twenty minutes in the dark can provide a measurement of q_T. At higher actinic light levels, chloroplast migration, q_M, acts as a light avoidance mechanism that affects fluorescence by decreasing leaf absorptance and increasing leaf transmittance. Cazzaniga (2013) found that it takes between 20 minutes and thirty five minutes for chloroplast migration to fully adjust. q_I or photoinhibition can also be determined with this peak, because F_M is known from the dark adapted first pulse and the difference is considered to be photo inhibition. Chronic photoinhibition starts to relax at about forty minutes in the dark and Lichtenthaler (1999, 2004) found that it can take up to 60 hours for complete relaxation of photoinhibition. It is common for researchers to dark adapt for 24 hours or overnight (Maxwell and Johnson 2000) when making this type of measurement.

It should be noted that NPQ should only be used to compare plants of the same species and with the same F_V/F_M values (Baker 2008), a (Maxwell and Johnson 2000).

Quenching relaxation measurements are valuable when using the lake model or the puddle model.

There is some overlap between photoprotective mechanisms q_E, Chloroplast migration q_M, (Cazzaninga 2013) and where they exist, with state transitions, q_T (Lichtenthaler 1999).

It is important to use a white stable actinic light source or a light source that allows intense blue light irradiation as well as red light to measure quenching parameters. Chloroplast migration is controlled by intense blue light, and it is relatively unaffected by intense red light (Cazzaniga 2013).These are measurements that are typically made after the leaf has reached steady state photosynthesis at a specific light level. If the light source is not stable, or if intense blue light is not used, measuring artifacts can occur.

8

After a saturation flash, the tailing off of the signal is the result of NPQ caused by the saturation flash. According to Rosenqvist and van Kooten (2006), it takes between one and two minutes for complete dissipation of saturation pulse NPQ. With this in mind, saturation pulses should be spaced to avoid build up of saturation pulse NPQ. It is also mentioned that photo-damage can occur to samples when saturation flash intensities are too high or too frequent when there is no actinic light. There is evidence that damage does not occur on samples in the light when high intensity saturation flashes are used even if they are used frequently (Rosenqvist 2006).

More Helpful Hints for Setting Test Variable in Quenching Protocols.

Saturation intensity

1. The saturation pulse should be intense enough to completely close all PSII reaction centers. To test this, one can try different saturation intensities and examine the saturation peak. This can be done in Excel software by using a curser to highlight only information in the trace data that is associated with the saturation pulse and several points on each side of a single saturation pulse. Use chart wizard and graph this highlighted data.

If the peak is rounded on the front and back of the pulse then the intensity is too low to fully saturate PSII. The intensity should be increased until the rounding disappears. There is evidence shows that very intense saturation pulses do not damage the plant in lighted conditions, but may damage plants in darkened conditions if they are too high and too frequent. (Rosenqvist and van Kooten 2006).

Under high actinic light, consider using the Multiflash protocol. The saturation flash always starts at 7,000 µmols and follows the Loriaux 2013 protocol. On the OS5p+ and the OS1p, Multiflash works in quenching protocols as well as with Y(II).

Saturation pulse frequency

2. By using the Stepped Actinic Test in the Kinetic Protocol, or quenching protocol, one can check to see if there is enough time between pulses to allow complete relaxation of saturation pulse NPQ.

In this case, all steps are set at one light intensity. The first step should include saturation pulses every two minutes apart for twenty minutes, or at high light intensities thirty five minutes. This will allow steady state leaf adjustment. Over the next six steps, the only variable that is changed is the time between saturation pulses. At each step shorten the time between pulses. If the F_M' values decline or the F_S (F') values increase, then there is not enough time for saturation pulse NPQ to relax. A spacing that prevents accumulation of saturation pulse NPQ is desirable to prevent measuring errors. When in doubt, use two minutes. Rosenqvist and van Kooten (2006) state that a time of between one and two minutes is required for complete relaxation of saturation pulse NPQ.

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