

Cookbook checklist before making light adapted Y(II) measurements. How accurate and repeatable is my Chlorophyll Fluorometer?

The above question is one of the most important and often asked questions regarding chlorophyll fluorescence, and is paramount to obtaining reliable data for your research. First let us define the basic terms.

Accuracy is the ability to hit the mark. In many types of measurements, accuracy is determined by calibrating to a measurement standard traceable to the National Institute of Standards and Technology (NIST). With such measurements, tolerances are always involved.

Y(II) is a measured normalized ratio, and accuracy is not determined by a traceable standard. Instead, it is determined by proper instrument usage in relation to plant physiology. Here, the standard has been developed by the research of experts in the field.

Repeatability is the ability to achieve the same measurement many times, within a specified tolerance level.

A **Reliable** measurement is one that is accurate and repeatable.

Chlorophyll fluorometers primarily measure normalized ratios that relate relative measurements of variable chlorophyll fluorescence found in Photosystem II. Many types of plant stress levels are reflected in the variable fluorescence of Photosystem II. This application note provides a cookbook style checklist of issues that must be considered in order to get reliable Y(II) measurements.

Y(II) or $\Delta F/F_m'$ is a measurement ratio 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). 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 light conditions in most land plants (Cazzaniga S. 2013). Photochemistry, heat dissipation, and chlorophyll fluorescence are competitive processes that compete for energy. Conditions that favor greater photochemistry cause lower chlorophyll fluorescence and heat dissipation. The relationship between Y(II) and photochemistry is linear in C₄ plants, and curvilinear in C₃ plant. In addition, photorespiration may limit measurement sensitivity issues in C₃ plants under drought stress, and other electron sinks may sensitivity for other types of plant stress.

First reported by Bernard Genty in 1989, this light adapted test became possible with the advent of modulated fluorometers. It is the most versatile plant stress measuring parameter, because it has been shown to detect more types of plant stress, earlier, than any other chlorophyll fluorescence method.

Cookbook checklist before making Y(II) measurements.

F_m' (maximum fluorescence in a light adapted environment at steady state photosynthesis). F_s' is the fluorescence signal in a light adapted environment at steady state photosynthesis.

Y(II) is = $(F_m' - F_s') / F_m' = \Delta F / F_m'$

1. Leaves must be at steady state photosynthesis. Under lower and medium light levels this takes between fifteen and twenty minutes at a given light level. Above canopy leaves on a clear day, in the field, are considered to be at steady state photosynthesis. (Maxwell and Johnson 2000). P 77. At high light levels steady state may take between *twenty minutes and thirty five minutes* due to chloroplast migration, a light avoidance mechanism that changes fluorescence parameters by changing leaf absorbance (Cazzaniga S. 2013).

2. It is dangerous to make Y(II) measurements on below canopy leaves in the field. The shade from higher leaves and wind can interrupt a plant's adjustment to steady state under ambient conditions. The xanthophylls cycle, and Δp of the thylakoid lumen adjust in about four to seven minutes, although it takes longer in the field. (Lichtenthaler 2004) (Baker 2004). State Transitions take between fifteen and twenty minutes to completely adjust. It has been found that state transitions are a big factor at lower light intensities, but they are not a factor at high light intensities. At near saturation light intensities can cause chloroplast migration, a mechanism that takes between 20 minutes and thirty five minutes to adjust to steady state photosynthesis. Rapid light curves and Fv/Fm may be better solutions for below canopy work where appropriate. The alternative is to use an internal fluorometer actinic light source, under a shroud, expose the sample to light for up to twenty minutes, to reach steady state, and then make a measurement.

3. Y(II) values vary with light level and with temperature. The higher the light level, the lower the Y(II) value. When measuring Y(II) in the field, it is extremely important to measure leaf irradiation or light level, at the leaf and leaf temperature. Comparing Y(II) values taken at different light levels and different temperature levels introduces a significant error, unless it is the change, at different light levels and heat levels, that is of interest. This is commonly done with a PAR Clip. (Genty 1989), (Genty 1990)

4. Shade leaves vs. Sun leaves. – The Y(II) ratio will be higher on sun leaves than on shade leaves (Lichtenthaler 2004).

5. Field plants should only be compared to field plants and green house plants should be compared to green houseplants due to light history. (Lichtenthaler 2004)

6. Leaf orientation. When making a yield measurement, with or without a PAR Clip, it is important not to change the orientation of the leaf. The leaf is at steady state photosynthesis in its current orientation. Changing the orientation changes the amount of light falling on the leaf, and the leaf will no longer be at steady state photosynthesis.

7. It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997)

8. The duration of the saturation pulse should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for Phytoplankton and cyanobacteria. Times outside these ranges increase the error in Y(II) measurements with most chlorophyll fluorometers. Shorter durations prevent complete saturation of PSII regardless of the light intensity (Roseqvist & van Kooten 2006). Longer durations create a form of saturation pulse NPQ that rounds the tail end of the pulse maximum value, and reduces the average maximum saturation pulse value (Roseqvist & van Kooten 2006). Some fluorometer allow adjustment of this parameter, and others are preset at the factory at either. 0.8 seconds, or 1.0 seconds for higher plants. 0.8 seconds is the default value on the OS1p and it will work well with almost all higher plants. The OS1p, the OS5p and the OS5p+ have a built rolling average capability to detect the highest eight point, 25 msec. rolling average. This prevents saturation pulse NPQ from being a problem if the duration is long enough. It can take up to 120 seconds for saturation pulse NPQ to fully dissipate, and so it is important to wait for at least two minutes between measurements at the same location.

9. Saturation pulse intensity. Saturation pulse intensity is more of an issue with Y(II) than with Fv/Fm. When dark adapting, shade leaves will saturate at a few hundred μmol s, and sun leaves will usually saturate below 1,500 μmol s. Indoor plants and under canopy plants saturate at much lower light intensities.

However, a problem has been found when measuring Y(II) at high light levels. It has been discovered that at high actinic or sun light levels, leaves resist the complete closure of all PSII reaction centers that is expected when using a saturation pulse. Even with a 7,000 μmol saturation pulse, some reaction centers remain open. Up to a 41% error was found in Y(II) measurements using standard techniques at high actinic light levels. To correct for this issue, multiple saturation flashes are used, and the measured maximum fluorescence value, for each flash, is entered into a linear regression analysis formula to determine the maximum fluorescence intensity, with an infinite saturation flash. The multiple saturation pulse approach has been shown to work in multiple papers and posters. The resulting value has been shown to correlate well with gas exchange carbon assimilation values. This multi-flash method is available on the OS5p+ and OS1p fluorometers. (see the Multi-flash application note for more details www.optisci.com). (Earl 2004) (Loriaux S.D., R.A Burns, Welles J.M., McDermitt D.K. Genty B. 2006) (Markgraf, T. and Berry J. 1990). The latest Multiflash protocol offered in the OS1p and the OS5p+ follow the protocol used in Loriaux 2013. Optimal setting are used.

10. PSI fluorescence - Part of the fluorescence signal contains PSI fluorescence as well as PSII fluorescence. With Y(II), one is trying to measure variable fluorescence of PSII in a light adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces a small error but it is not a problem for comparing similar samples, because PSI fluorescence does not change with light intensity temperature or plant stress. (Baker, Oxborough 2004)

11. “Super-saturating flash” error is produced by using a very intense saturation light source that is longer than 2ms causing multiple turnovers of primary PSII receptor Q_A and the reduction of plastoquinone to plastoquinol. This raises F_m (or F_m') and can cause an overestimate of Yield by less than 10% (Baker and Oxborough 2004), (Schreiber 2004). Use of a super-saturation flash is by far the most common method of measuring yield in higher plants. As long as one is interested in plant stress and not exact correlation to CO_2 carbon assimilation this is not an issue.

12. Cold stress can produce a non-linear correlation with CO_2 assimilation. Electron transport of PSII in cold stressed corn far exceeds the requirements for CO_2 assimilation by more than three to one, indicating that under these conditions, other electron sinks are at work. The ratio of ETR (a product of Y(II), PAR, leaf absorption ratio, and PSII absorption ratio) to CO_2 assimilation, under cold stress, can be diagnostic for cold stress. (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998)

13. The ratio of ETR to CO_2 assimilation can be diagnostic for water stress in C_3 plants. C_3 plants exhibit strong electron transport rates for early and moderate levels of water stress even when CO_2 assimilation has decreased due to water stress. This indicates that there are other electron sinks for electron transport. (Ohashi 2005). *This problem of early water stress measurement and detection may be overcome by using a special assay discussed in Burke 2007 and Burke 2010.*

14. Mangrove leaves growing in the tropics. Here again electron transport rate is more than three times that of CO_2 assimilation. It is believed that this is mostly due to reactive oxygen species as an electron sink. (Baker Oxborough 2004), (Cheeseman 1997)

15. While linear correlation occurs between Y(II) and ETR with CO_2 assimilation in C_4 plants and curvilinear correlation between Y(II) and ETR with CO_2 assimilation in C_3 plants, (Genty 1989), (Genty 1990), (Baker Oxborough 2004), *exact* correlation between fluorescence ETR and gas exchange carbon assimilation is not possible due to the fact that fluorescence comes from only the upper most layers of the leaf while gas exchange measurements measure lower layers as well (Schreiber 2004).

16. Chlorophyll fluorescence Heterogeneity – Chlorophyll fluorescence can vary from one part of a leaf to another and become patchy under certain circumstance. Under drought stress, cold stress, or CO_2 stress it is best to take multiple leaf measurements and average the values (Baker 2008)

17. Light history – It takes between forty minutes and sixty hours for chronic photoinhibition to relax or repair in a leaf. Since photoinhibition reduces chlorophyll fluorescence measuring parameters, it is important to compare samples that have a similar recent light history. There will be some residual photoinhibition after a bright summer day and there may be no residual photoinhibition after a few overcast days (Lichtenthaler 2004).

PAR is photosynthetically active radiation. Radiation on the leaf is measured between the wavelengths of 400nm to 700 nm. PAR sensors and thermistors for measuring temperature are calibrated to other instruments that are traceable to the NIST. It is recommended that recalibration should occur every two years. Most modern sensors are solid state, so drift is minimal.

Y(II) is sensitive to most types of plant stress. We have listed some important notes below. For more information request the Opti-Sciences Plant Stress Guide and quantum photosynthetic yield application note at www.optisci.com

1. Y(II) and ETR are sensitive to drought stress in C₄ plants. (da Silva J. A. & Arrabaca M.C. 2004) (J Cavender-Bares & Fakhri A. Bazzaz 2004) (Cerovic 1996)
2. Y(II) in the *Burke assay*, is very sensitive to drought stress in C₃ plants. (Burke 2007, Burke 2010) Drought stress can be detected within 24 hours or irrigation cessation. This is a special assay that included yield measurements. It may be used for large or small populations of plants.
3. Y(II) *by itself is not sensitive to drought stress* until it is relatively advanced.
4. Y(II) is Sensitive to heat stress above 35 degrees centigrade in Oak, a C₃ plant. (Haldiman P, & Feller U. 2004)
5. Y(II) is not sensitive to sulfur stress until starvation levels are reached. (Baker 2004)
6. Y(II) is not sensitive to early or moderate CO₂ stress. (Siffel & Braunova 1999)
7. Y(II) is not sensitive to NaCl stress in Rice, but it is sensitive to NaCl stress in sorghum and chickpea.(Moradi & Ismail 2007) (Netondo 2004) (Eyidogan 2007)

F_V/F_M is not as sensitive to drought stress, heat stress, nitrogen stress, sulfur stress, nickel stress, zinc stress, some types of chemical stress, and some types of herbicide stress. For more information about specific types of plant stress request the Opti-Sciences Desk Top Plant Stress Guide at www.optisci.com.

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