Fluorometry – made simple

Introduction

Advances in instrumentation over the last two decades have made possible non-intrusive means of examining the kinetics of photosynthesis (however, see limitations imposed by insufficient water motion in the Discussion section). A notable advance has been that of modulated pulse fluorometry. One of these instruments, a **pulsed amplitude modulation (PAM) fluorometer**, examines chlorophyll fluorescence and is able to determine how energy is used (and not used) by photochemical reactions. In essence, a PAM chlorophyll fluorometer is a 'photosynthesis meter,' and allows one to gain insights of photochemical and non-photochemical reactions.

Chlorophylls are abundant photopigments and, along with accessory or antennae pigments, harvest light energy. By-products of molecular oxygen and organic carbon are ultimately produced through the process known as photosynthesis.

If a form of chlorophyll - chlorophyll *a* - is exposed to strong light, it will absorb a portion of the light's energy and use it in photosynthesis. Chlorophyll *a* will also absorb and emit some of this light's energy at a lower energy level in a phenomenon known as fluorescence. Fluorescent emissions of chlorophyll *a* are generally considered red, and are known to range from ~660 nm to ~760 nm. Plants, marine algae, and corals with healthy symbiotic dinoflagellates will fluoresce when exposed to relatively high amounts of visible light. Chlorophyll fluorescence is proportional (to a point) to the amount of photosynthetically active radiation. If no light energy is available for, say, 20 minutes, chlorophyll fluorescence is, for all intents and purposes, at zero, so a very weak amount of light (< 1 $\mu mol \cdot m^2 \cdot sec$) is applied by a PAM meter to cause chlorophyll *a* to weakly fluoresce. This is measured and reported as Minimum Fluorescence (**Fo**). If a brief pulse of intense, photosynthetically saturating light is applied to a *dark-adapted* sample, the fluorescence will rise to a maximum level. This is called Maximum Fluorescence (**Fm** - See Figure 1). It is also possible to estimate 'variable' fluorescence (**Fv**) simply by subtracting **Fo** from **Fm.** Fluorescence of an *illuminated* sample during a saturating pulse of light when all PS II reaction centers are saturated with light ('closed'), is called Maximum Fluorescence (**Fm'** - the prime symbol indicates an illuminated sample). **Fm'** is generally less than **Fm**. If one were to subtract **Fm'** from **Fm**, the difference is due to 'non-photochemical reactions' (denoted as **qN** or **NPQ**, depending upon circumstances). Non-photochemical reactions compete with photochemical reactions in 'quenching' (suppressing) maximum fluorescence. Thus, results from measurements of 'minimum,' 'variable' and 'maximum' fluorescence can be manipulated mathematically to determine how light energy is used and/or dissipated.

Note: This fluorometer uses a 'red' light-emitting diode (LED) as 'actinic' light. This light energy is absorbed by PS II's reaction center, containing Pigment 680 (P-680). Since P-680 (a specialized form of chlorophyll *a*) absorbs energy collected by chlorophylls *a*, c_2 and accessory pigments such as peridinin, the red excitation bandwidth is appropriate for use with algae. A light filter (cutoff λ <680 nm) prevents the PAM's internal photo-amplifier from confusing excitation wavelengths for those of chlorophyll fluorescence. There are some advantages (and disadvantages) of using a 'blue' LED as an actinic source, however, these do not dismiss the results gathered while using red wavelengths as an excitation source.

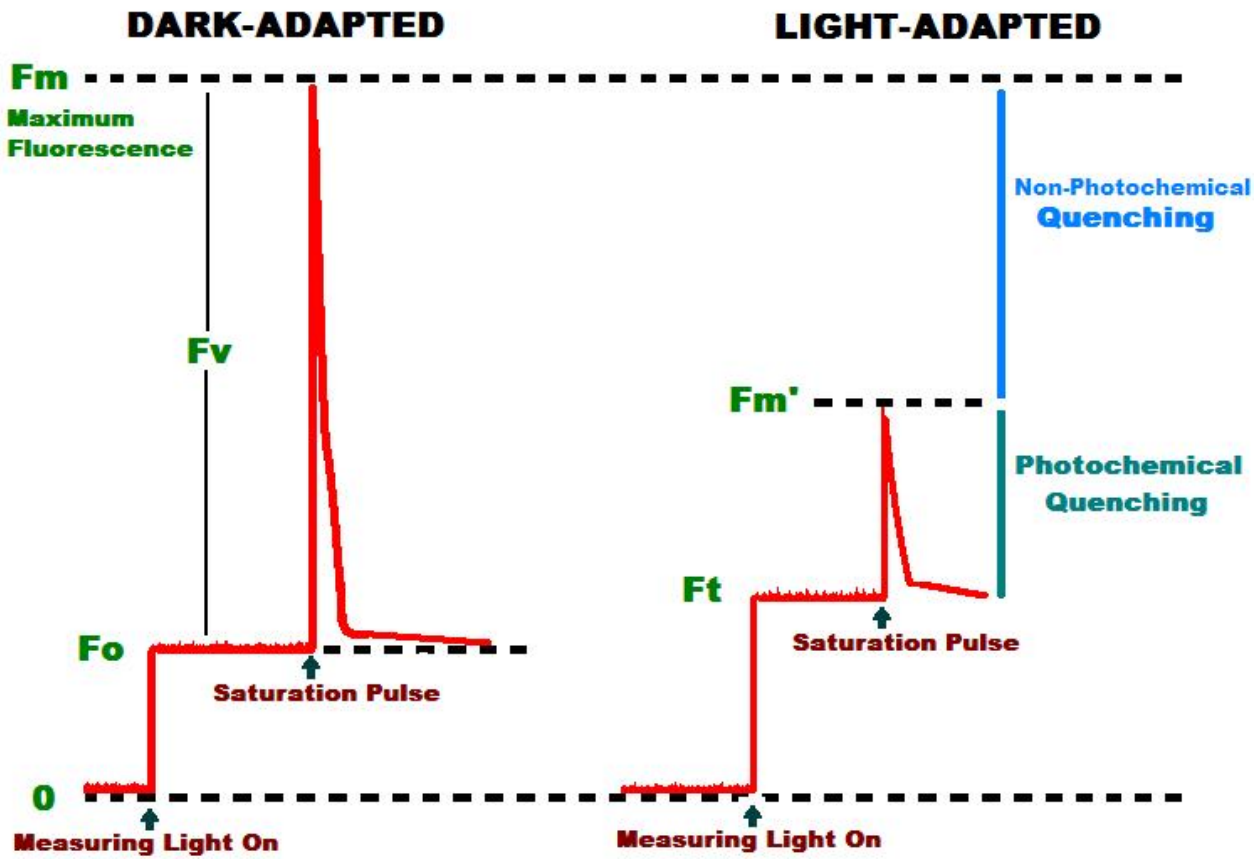


Figure 1. Chlorophyll fluorescence. After Schreiber, 1997.

These formulae are used to determine photochemical efficiencies:

Photochemical Quenching (qP): (Fm'-Ft)/ (Fm'-Fo). **qP** is the energy absorbed by PS II.

Non-photochemical Quenching (qN): (Fm-Fm')/ (Fm-Fo). **qN** is generally associated with non-photochemical activity, such as dissipation of absorbed energy as heat, or as a thylakoid-energizing prelude to photosynthesis.

***Non-photochemical Quenching (NPQ)*:** (Fm-Fm')/Fm'. **NPQ** is particularly associated with energy dissipation as non-radiant heat through the 'xanthophyll cycle.'

***Yield of Photochemical Energy Conversion*:** (Fm'-Ft)/Fm' = ΔF/Fm'

Light-harvesting Structures of Algae

Light-harvesting photopigments within algae are found in thylakoid membranes contained within structures called chloroplasts (See Figure 2). It is thought that one Photosystem I and

one Photosystem II are arranged within a few microns of each other on the thylakoid, so that they may act effectively transfer energy. These photosystems combined are known as a Photosynthetic Unit, or PSU (Kirk, 2000).

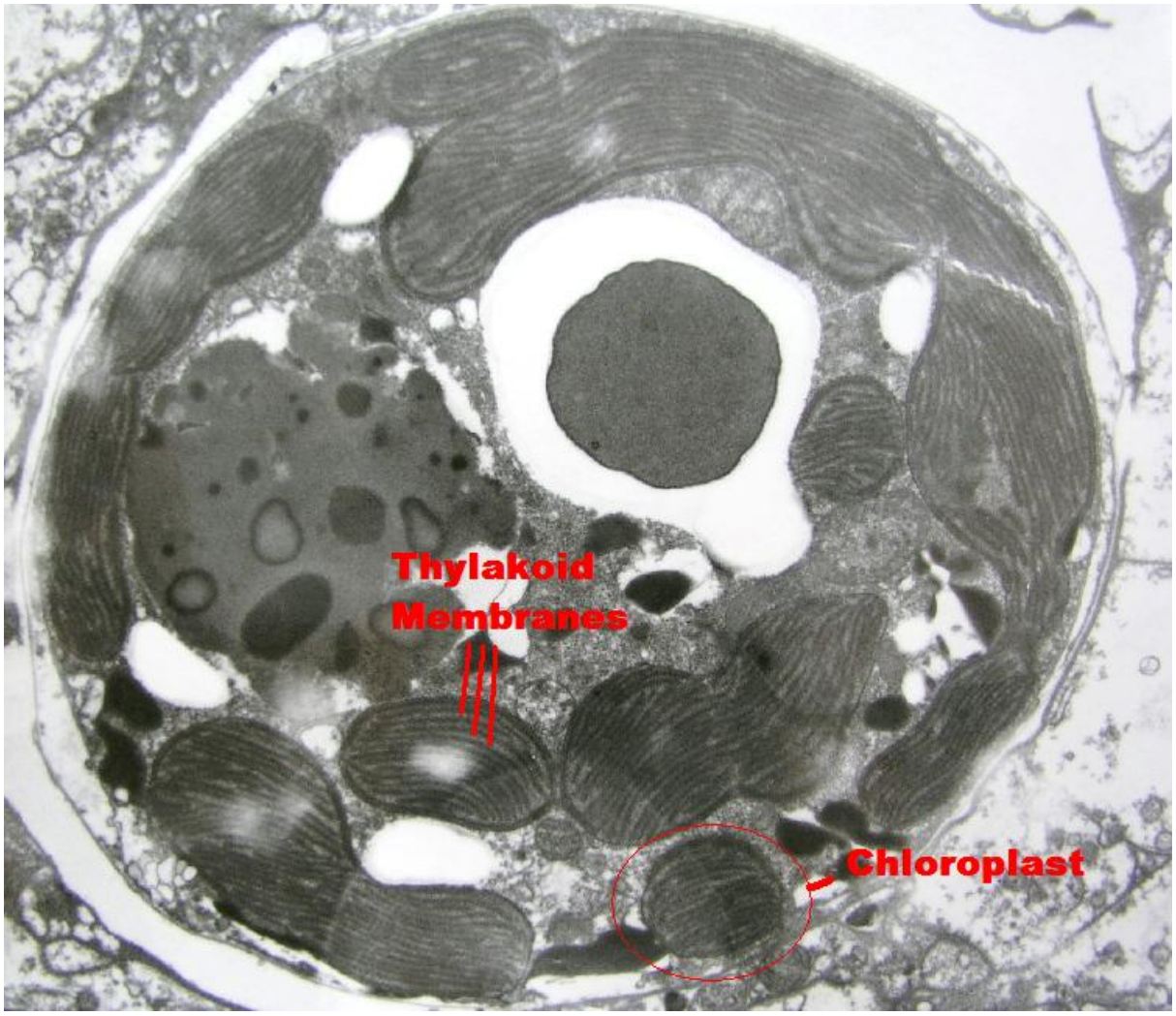


Figure 2

An electron photomicrograph of a algae. Chloroplasts containing thylakoid membranes are major structures within the dinoflagellate. *Photo courtesy of Dr. Dennis Aaron and Dr. Steven Poet, University of Georgia, College of Veterinary Medicine.*

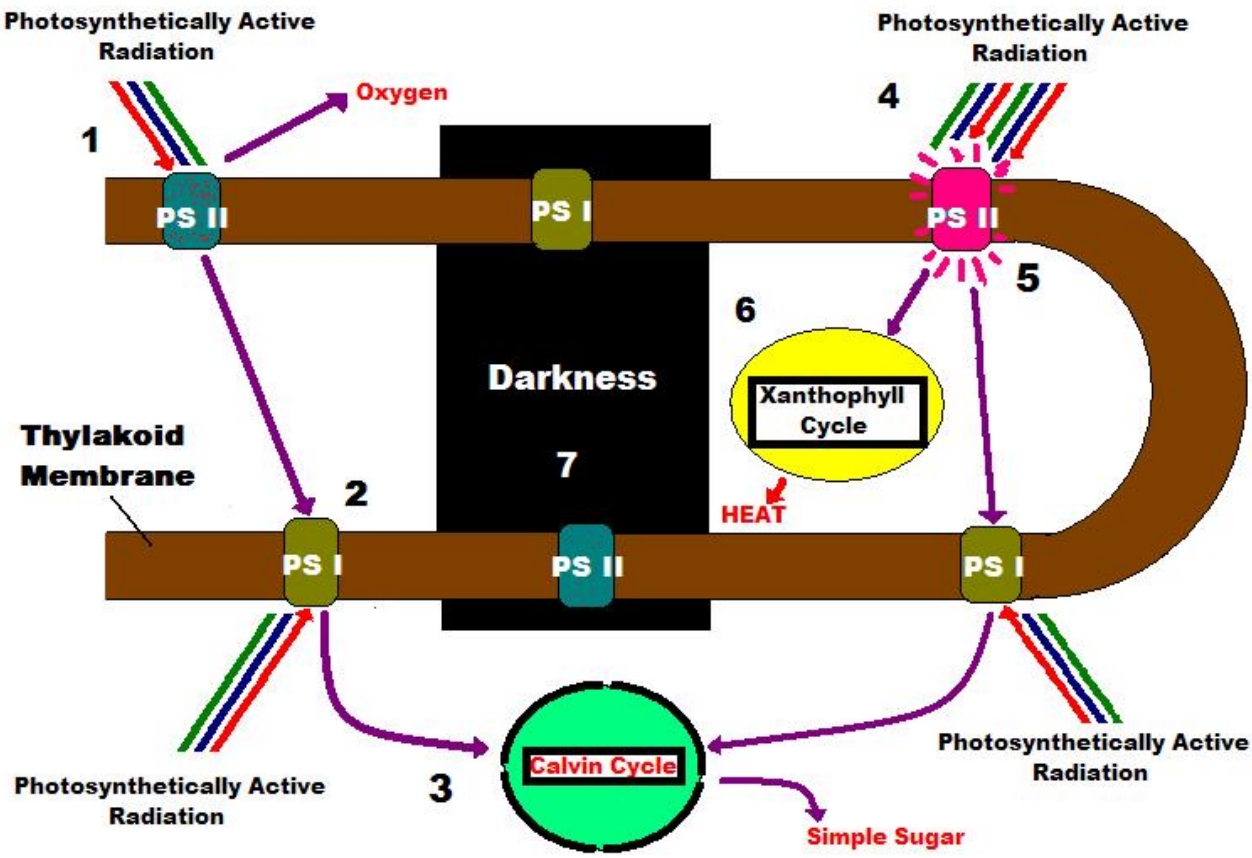


Figure 3. A Simple Schematic of Photosynthesis

1. A moderate amount of light (or PAR - photosynthetically active radiation) falls upon one of the many thylakoid membranes (brown line) containing a Photosynthetic Unit (consisting of one Photosystem II and one Photosystem I) within an alga. Photosystem II (PS II) photopigments absorb PAR and transfer its energy to a 'Reaction Center' within PS II. Note that some variable chlorophyll *a* fluorescence occurs even at moderate light intensity, as Reaction Centers absorb light and begin to 'close.' One oxygen molecule is created for every two water molecules split.

2. Light energy collected by PS II is transferred to PS I (specifically the PS I Reaction Center). PS I photopigments also collect PAR transfers energy to:

3. The Calvin Cycle, where inorganic carbon is converted to simple sugar.

4. Under conditions of high PAR intensity, 'safety valves' for excess light energy come into play. PS II reaction centers absorb as much energy as they can (photosynthesis is said to be 'saturated' when all reaction centers are 'closed'), and two safeties dump excess energy:

5. *Chlorophyll fluorescence*, which depends upon the number of 'closed' reaction centers and:

6. Transfer of energy to the '*Xanthophyll Cycle*,' where energy absorbed by the antennae pigments is dissipated as non-radiant heat, and involves the reversible, light-mediated conversion of diadinoxanthin to diatoxanthin within algae.

7. In darkness, PS II chlorophyll fluorescence is at a minimum, and no photochemistry occurs. However, the Xanthophyll Cycle continues with conversion of diatoxanthin back to diadinoxanthin.

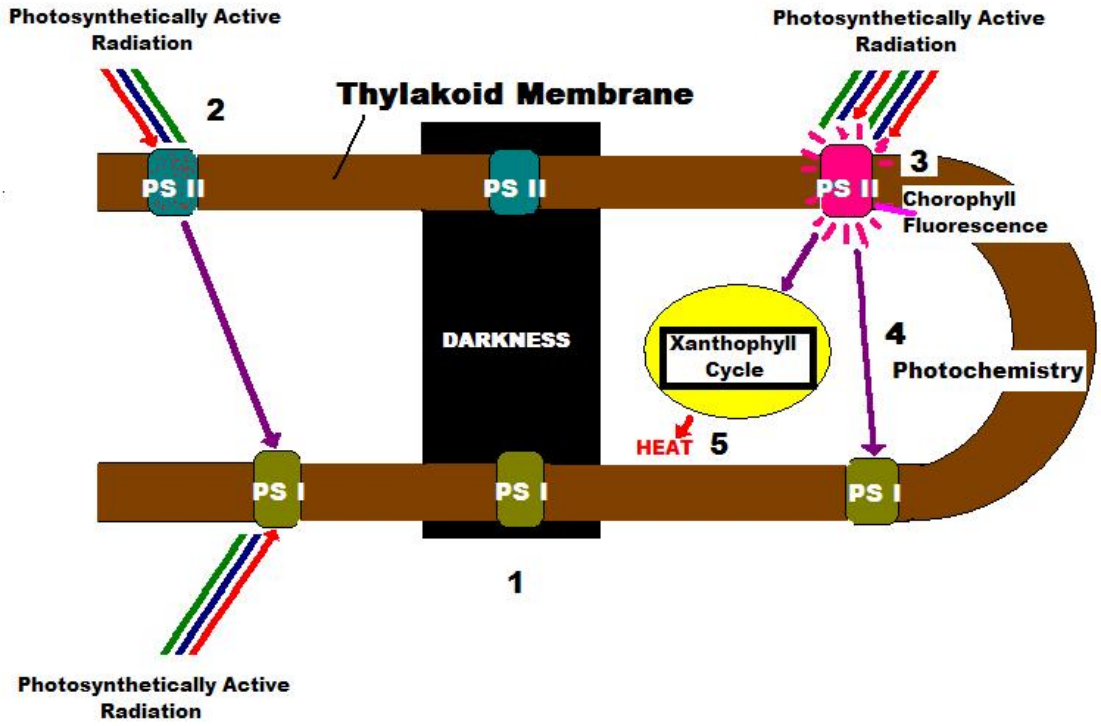


Figure 4. Terminology of PAM Fluorometry and Definitions.

1. In darkness, minimal fluorescence of PS II chlorophyll is found. In other words, no energy is available to the photosystem, and it is fully 'open' (oxidized) and ready to absorb light energy. A small amount of light energy is applied to the sample, and this induces chlorophyll fluorescence, known as **Minimal Fluorescence Yield** *following* ***dark adaptation***, and noted as **Fo**. If a saturating pulse of light is applied to the dark-adapted sample, the Reaction Centers are 'closed' (reduced) and chlorophyll fluorescence will be at its highest value. **Maximum Fluorescence** of a dark-adapted sample is called **Fm**.

2. When illuminated with non-saturating light intensity, the chlorophyll molecules of PS II begin to fluorescence as Reaction Centers are reduced (closed). This fluorescence is known as **Variable Fluorescence** (**Fv**, which is equal to Fm - Fo - or Fm' - Fo', see below).

3. The **Maximum Fluorescence** of the illuminated sample is known as **Fm'**.

4. Under conditions of proper illumination (and other environmental conditions), the **Electron Transport Rate** (ETR) will continue between Photosystems I and II. Photosynthesis is said to be 'saturated' when the amount of PAR available to the photosystems meets or exceeds the maximum rate absorbed by the photosystem, and increasing the amount of light will not increase the rate of photosynthesis. A method of energy dissipation - other than

photochemical quenching (i.e., the absorption of energy used in photochemistry and called **qP**) - must then be used, and this is known as:

5. 'Non-photochemical quenching' or **NPQ**. NPQ involves the 'Xanthophyll Cycle' where protective pigments dissipate excess harvested photons as non-radiant heat.

Glossary

Fo = Minimal Fluorescent Yield after dark adaptation.

Fo' = Minimal Fluorescent Yield of illuminated sample.

Fm = Dark-adapted Maximal Fluorescent Yield reached with a saturating pulse of light.

Fm' = Light-adapted Maximal Fluorescent Yield reached with a saturating pulse of light.

Ft = Fluorescent Yield at a given time, generally just before a saturation pulse is applied to a sample.

Fv = Variable Fluorescence (Fm - Fo or Fm'-Fo').

Fv:m or **Fv:Fm** or dark-adapted yield = Maximal Quantum Yield of a dark adapted sample and equals (Fm-Fo/Fm).

qP (photochemical quenching) = (Fm' - Ft)/ (Fm'-Fo). Photochemical fluorescence quenching is indicative of the proportion of PAR absorbed by the 'open' reaction centers of PS II and hence used in photochemistry. This coefficient may vary between 0 and 1.

Chronic Photoinhibition = Photoinhibition is characterized by a type of non-photochemical quenching which recovers only slowly (if at all) in the dark.

Dark Adaptation = A brief (usually 30 minute) acclimation time in darkness. During this time, photochemical reactions stop, and all reaction centers 'open' to receive light energy when it becomes available.

Dynamic Photoinhibition = The same as NPQ: The quenching of fluorescence by dissipation of excess light energy as heat. Involves xanthophylls.

Saturation - Maximum photosynthetic rate or photosynthetic capacity.

Thylakoid - A lipid membrane within a chloroplast that contains photopigments comprising PSI and PSII.

Yield (light adapted) = Quantum Yield of photochemistry PS II, measured on light adapted samples. (Fm'-Ft)/Fm' (or $\Delta F/Fm'$).

Yield (dark adapted) = Quantum Yield of photochemistry in PS II, measured on dark adapted samples. (Fm - Fo/Fm).

Zero Offset = This number represents a background signal found within the instrument. Abbreviated as 'Zoff', it is automatically subtracted from Ft, and all consequently determined fluorescent values.

qN (non-photochemical quenching) = (Fm-Fm')/(Fm-Fo) or, alternately, (Fm-Fm')/Fm-Fo'). This coefficient may vary between 0 and 1. However, if qN exceeds ~0.4 there is also significant quenching of Fo, and NPQ should be examined.

Hence, qN = (Fm-Fm')/ (Fm - Fo'). Note: This formula has also been used for qN (qN = 1 - (Fm' - Fo')/(Fm - Fo) = 1 - Fv' : Fv), and provides values very close to that immediately above. Useful only when photosynthesis is activated, usually after ~ 2 minutes of illumination.

NPQ (Nonphotochemical quenching or Nonphotochemical exciton quenching - Kanazawa and Kramer, 2002). NPQ = (Fm-Fm')/Fm'. NPQ can vary between 0 and infinity, but, for practical purposes, is unlikely to exceed a value of 10. The choice between NPQ and qN depends upon the application - with NPQ, that part of photochemical quenching is emphasized that reflects heat-dissipation of excitation energy in the antennae system. (Hence, NPQ is a convenient indicator of 'excess light energy' - Schreiber, 1997). NPQ is relatively insensitive to that part of non-photochemical quenching which is associated with qN values between 0 and 0.5. Nonphotochemical quenching of excitation energy, which protects higher plant photosynthetic machinery from photodamage, is triggered by acidification of the thylakoid lumen as a result of light-induced proton pumping, which also drives the synthesis of ATP. In essence, excess absorbed light energy is dissipated as heat within the light-harvesting complexes. NPQ involves two processes activated by the acidification of the lumen, the interconversion of xanthophyll cycle carotenoids, and the protonation of residues on key LHC components. In *absence* of NPQ modulation, buildup of reduced electron carriers would block electron flow before the lumen could be significantly acidified. This over-reduction could result in the formation of a stable, doubly-reduced Qa species in PS II, allowing the formation of triplet chlorophyll species, which in turn can react with O2 to form singlet oxygen ($^1O_2$), an extremely toxic oxygen radical.

ETR (electron transport rate) = Effective quantum yield (Fm' - Ft)/Fm' X PAR. Ralph, Gademann, Larkum and Kühl (2002) believe Beer et al. (1998) underestimated absorption coefficients of corals (measured as 0.023 - 0.036, as compared to 0.86 for terrestrial green leaves). Hence, Ralph et al. recommend reporting 'Relative ETR', as determined by the above formula, until a widely accepted method of determining absorption coefficients is established.

Xanthophylls = Oxygenated carotenoid pigments produced by plants. Xanthophylls are anti-oxidants and may help detoxify oxygen radicals.

Some are also involved with energy dissipation which involves light mediated changes to their structures.

References

1. Atkinson, M. and C. Bingman, 1999. The composition of several synthetic seawater mixes. Aquarium Frontiers Online. March, 7 pp.
2. Atkinson, M.J., E. Kolter and P. Newton, 1994. Effects of water velocity on respiration, calcification and ammonium uptake of a *Porites compressa* community. Pac. Sci., 48(3):296-303.
3. Atkinson, M.J., B. Carlson and G.L. Crow, 1995. Coral growth in high nutrient, low-pH seawater: a case study of corals cultured at the Waikiki Aquarium, Honolulu, Hawaii. Coral Reefs.
4. Atkinson, M.J. and R.W. Bilger, 1992. Effects of water velocity on phosphate uptake in coral reef-flat communities. Limnol. Oceanogr., 37(2):273-279.
5. Beer, S., M. Ilan, A. Eschel and I. Brickner, 1998. Use of pulse amplitude modulated (PAM) fluorometry for *in situ* measurements of photosynthesis in two Red Sea faviid corals. Marine Biology, 131: 607-612.
6. Bongiorni, L., Shafir, S., Angel, D. and B. Rinkevich, 2003. Survival, growth and gonad development of two hermatypic corals subjected to in situ fish-farm nutrient enrichment. **Marine Ecology Progress Series 253:137-144**.
7. Falkowski, P. and Z. Kolber, 1995. Variations in chlorophyll fluorescence yields in phytoplankton in the world's oceans. Aust. J. Plant Physiol., 22: 341-355.
8. Fitt, W. and M. Warner, 1995. Bleaching patterns of four species of Caribbean reef corals. Biological Bulletin (Woods Hole), 187, 298-307.
9. Gorbunov, M., P. Falkowski and Z. Kolber, 2000. Measurement of photosynthetic parameters in benthic organisms in situ using a SCUBA-based fast repetition fluorometer. Limnol. Oceanogr., 45(1), 242-245.
10. Gulko, D., 1998. *Hawaiian Coral Reef Ecology*. Mutual Publishing, Honolulu. 245 pp.
11. Hall, D. and K. Rao, 1999. *Photosynthesis*. Cambridge University Press, Cambridge. 214 pp.
12. Hoegh-Guldberg, O., 1999. Climate change, coral bleaching and the future of the world's reefs. Mar. Freshwater Res., 50, 839-866.
13. Jones, R., O. Hoegh-Guldberg, A. Larkum and U. Schreiber, 1998. Temperature-induced bleaching of corals begins with impairment of the CO2 fixation mechanism in zooxanthellae. Plant, Cell and Environment. 21, 1219-1230.
14. Kanazawa, A. and D.M. Kramer, 2002. *In vivo* modulation of nonphotochemical quenching (NPQ) by regulation of the chloroplast ATP synthase. Proc. PNAS, 99(20): 12789-12794.
15. Kirk, J.T.O., 2000. *Light and Photosynthesis in Aquatic Ecosystems*. Cambridge University Press, Cambridge. 509 pp.
16. Kolbert, O. Prasil and P. Falkowski, 2000. Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: Defining methodology and experimental protocols. Biochim. Biophys. Acta., 13 (67): 88-106.
17. Ralph, P.J., R. Gademann, A.W.D. Larkum and M. Kühl, 2002. Spatial heterogeneity in active chlorophyll fluorescence and PSII activity of coral tissues. Marine Biology, 141: 639-646.
18. Schreiber, U., 1997. *Chlorophyll Fluorescence and Photosynthetic Energy Conversion*. Heinz Walz GmbH, Effleltrich. 73 pp.

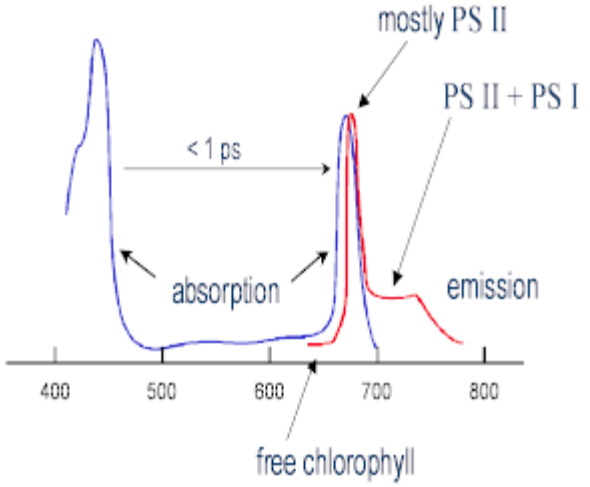
Multiple-, Single turnover and Fast Repetition rate (FRRF)

From:
http://www.chelsea.co.uk/Instruments%20FASTtracka.htm#Background
and http://www.chelsea.co.uk/LFRRFII

General principles

Active chlorophyll *a* (Chl *a*) fluorometry provides a non-destructive and minimally intrusive method for probing oxygenic photosynthesis, in general, and the functioning of photosystem II (PS II), in particular.

Within PS II, Chl *a* fluorescence competes directly with photochemistry and non-radiative decay for excitation energy. By measuring Chl *a* fluorescence, it is possible to gain information about the other two processes.



The peak in the Chl *a* fluorescence emission spectrum at 683 nm is 'Stokes shifted' from the absorption peak that is associated with the S1 excited state of Chl *a* and is dominated by the contribution from PS II.

Chl *a* is present within both PS II and PS I. The FAST*tracka* II defines the Chl *a* fluorescence signal using a narrow bandpass filter, centred at 685 nm, which minimises contamination of the PS II signal from PS I and free Chl (see figure above).

The Multiple Turnover (MT) method

Fluorimeters that employ the multiple turnover method use a relatively long saturating pulse of high photon irradiance (PI) to drive the yield of photochemistry close to zero, through multiple turnovers of PS II: typically, a few hundred ms at several thousand µmol photons m^{-2} s^{-1}. By comparing the fluorescence signal before the pulse is applied (F_o or F' with dark or light-adapted material, respectively) with the highest fluorescence signal achieved during the saturating pulse (F_m or F_m' with dark or light-adapted material, respectively) it is possible to estimate the yield of PS II photochemistry, as 1 - F_o/F_m or 1 – F'/F_m' (often written as F_v/F_m or F_q'/F_m', where F_v and F_q' are $F_m – F_o$ and $F_m' – F'$, respectively).

The multiple-turnover method has a number of weaknesses:

- A multiple-turnover measurement typically takes several hundred ms. When profiling or attached to a towed vehicle, this is very likely to mean that the multiple-turnover pulse is spread over a long trail of sample, such that F_m or F_m' is never reached. This could result in a very significant underestimate of PS II photochemical efficiency.
- Photosynthesis takes tens of seconds to 'recover' from the application of a multiple-turnover pulse. This limits the frequency at which measurements can be taken from a single sample .
- No information about the absorption cross-section of PS II can be derived from multiple-turnover data.

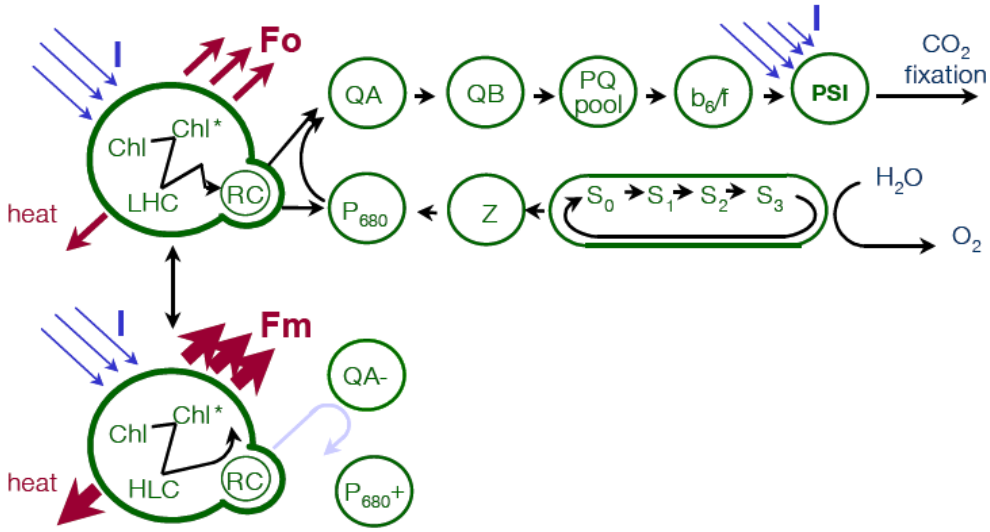
The Single Turnover (ST) method

Fluorimeters that employ the single-turnover method use a very short saturating pulse of very high PI to drive the yield of photochemistry close to zero, through near-simultaneous turnover of all PS II centres: typically, 100 µs at 20 000 µmol photons m^{-2} s^{-1}. The yield of PS II photochemistry can then be calculated, in the same way as with the multiple-turnover method.

The Fast Repetition Rate (FRR) technique

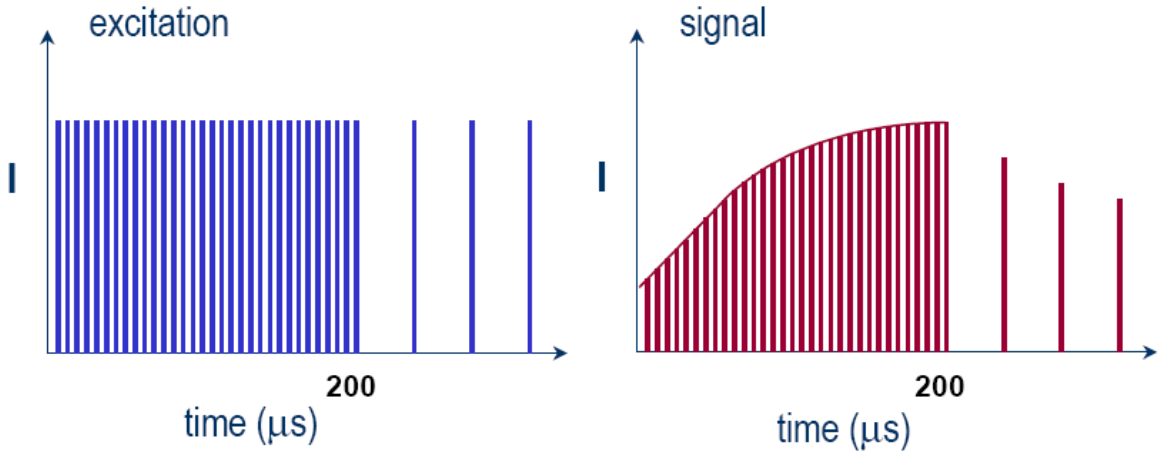
SCIENTIFIC PRINCIPLES

A schematic of the photosythesis process that occurs in aquatic microorganisms is illustrated schematically below:

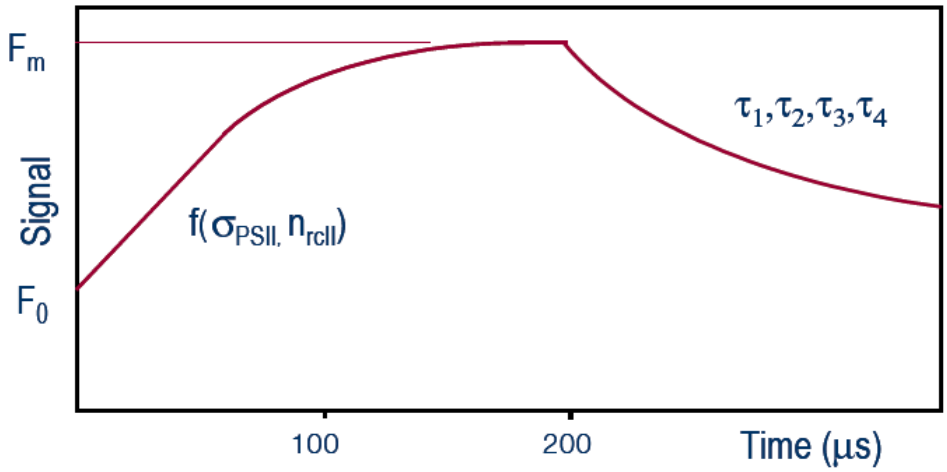


The light-harvesting complex (LHC) first absorbs ambient light (I) and the energy is transferred to the reaction centre (RC) through via a number of fluorescence energy transfer steps between chlorophyll molecules. On absorbing this energy the reaction centre initiates a cycle of photosynthesis, which takes approximately 200µSecs to complete. During this time the reaction centre remains 'closed' and is unable to absorb any more energy from the light-harvesting complex, this excess energy is then dissipated as heat or additional fluorescence.

In FRRF a rapid series of 1µSec light pulses, typically 100 pulses spaced 1µSec apart, is directed into the water sample. Each pulse closes a proportion of the reaction centres and the detected fluorescence signal increases from an initial value (F_0) to a maximum value (F_m) when all the reaction centres have been closed. This time-dependent fluorescence signal can only arise from organisms that are photosynthetically active; chlorophyll that is either free in solution or present in dead organisms may change the value of F_0 but will not produce this so-called 'variable fluorescence'.



The magnitude and shape of the response (see diagram below) can be used to derive a number of important parameters that relate to the health of these organisms and their photosynthetic activity and because of this any chemical pollutants that affect the photosynthetic process are likely to be detected using FRRF.



$(F_m - F_0)/ F_m$: photochemical quantum efficiency (F_v/F_m)

σ_{PSII}: efficiency of light harvesting (m^2 photon^{-1})

n_{rcII}: concentration of active reaction centres

τ_i: rate constants of electron transfer from PSII to PSI

The most important parameter obtained from FRRF is the variable fluorescence expressed as a fraction of the maximum signal, known as Fv/Fm. This parameter is related to the photochemical quantum efficiency and can vary from very low values for organisms in a poor environment to a maximum value of around 0.65 for those in an optimal one. It is likely that pollutants or toxins will have a direct impact on this parameter and importantly, because the value is expressed as a ratio, it is independent of chlorophyll concentration. Other parameters that can be obtained from the variable fluorescence response are the concentration of reaction centres (n_{rcII}) and the absorbance cross-section of the light harvesting complexes ($\int_{PSII}$). For example, in nutrient rich environments where there is plenty of light, the light harvesting complexes will undergo conformational changes that optimise their light collection efficiency and the absorbance cross-section will increase..

The implementation of the FRR technique within the FAST*tracka* II sensor maximises both the accuracy and the amount of information that can be derived from the Chl *a* fluorescence signal, by incorporating the following design features:

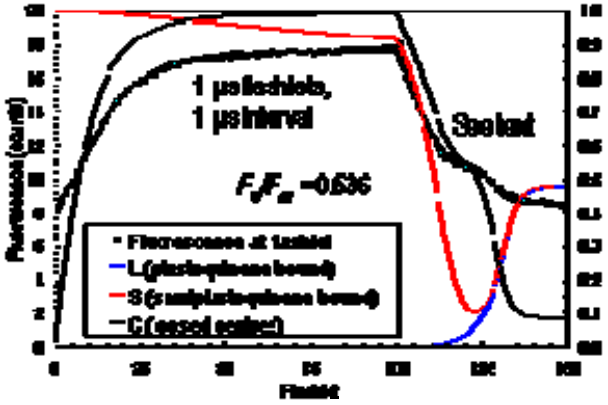
- An array of short (1 µs) measuring 'flashlets' are used both to excite Chl *a* fluorescence and provide the actinic illumination required to achieve F_m or F_m', by driving the yield of PS II photochemistry close to zero.
- The fluorescence signal is integrated over the entire duration of each flashlet.
- The output from the LEDs is also integrated over the entire duration of each flashlet .

Any drift in the sensitivity of the photomultiplier tube used to record the Ch a fluorescence signal, or the photodiode used to monitor the output from the LEDs, is tracked by sampling between flashlets.

Overall, this approach maximises the signal to noise ratio, whilst minimising measurement artefacts.

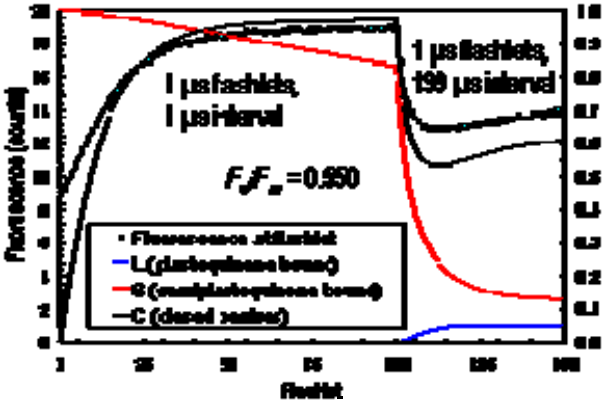
Typically, an FRR ST saturation phase is formed of 100 x 1 µs flashlets of 30 000 µmol photons m^{-2} s^{-1}, with a 1 µs interval between adjacent flashlets, whilst an FRR MT saturation phase is formed of 3200 flashlets of 1 µs duration with 49 µs between flashlets. The sensitivity of the photomultiplier tube and the output from the excitation LEDs is monitored at each flashlet, to minimise measurement artefacts. Implementation of the ST FRR method provides information about PS II, over and above that provided by all other methods. Specifically, the level of connectivity among PS II complexes (*p*), and the 'effective' absorption cross section of PS II ($\sigma_{PS\,II}$) which is required for calculation of the electron transfer rate through each PS II complex ($ETR_{PS\,II}$). $ETR_{PS\,II}$ is often used in estimations of primary productivity.

Sample Data

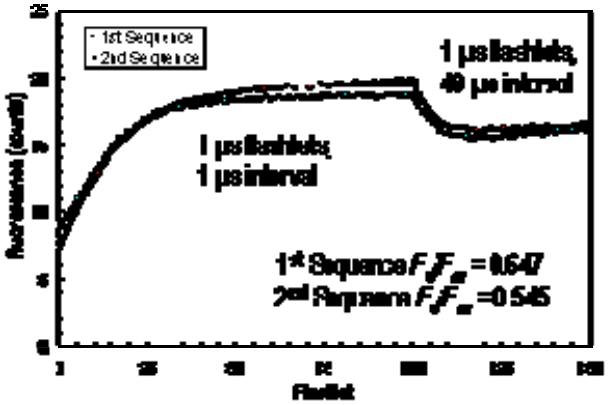


Data fit to the mean of sequences 2 to 6 of a 6 sequence acquisition from dark-adapted material. A mechanistic model (currently under development), which incorporates the turnover of plastoquinone, semi-plastoquinone and plastoquinol at the Q_B site of PS II, was used to fit these data.

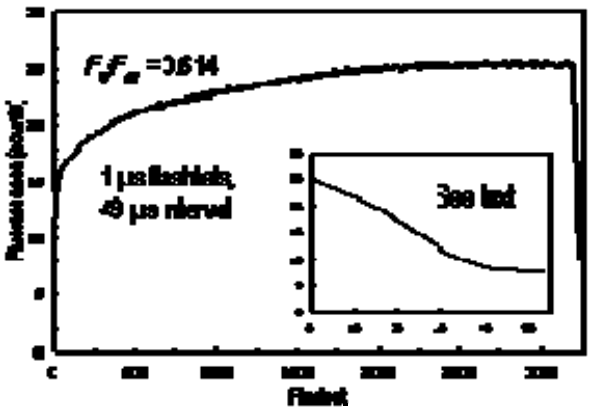
The interval between flashlets during the relaxation phase started at 50 µs, but was increased by 20% between consecutive flashlets. The model has generated a reasonable fit to the data, including the 'kink' in the relaxation phase.



Same as above except that the interval between flashlets during the relaxation phase was fixed at 199 µs. The mechanistic model has produced a reasonable fit to the data, including to the slow fluorescence increase during the latter part of the relaxation phase.



Analysis of individual sequences within an acquisition. To generate these data, six acquisitions of two sequences were made from dark-adapted cells, with a 30s intervals between acquisitions. The mean trace of the first sequences shows a lower value for F_o, but higher values for σPS_{II} and *Fm* than for the second sequence. The higher *Fm* value for the first sequence in an acquisition from dark-adapted material is a common feature of ST data. For example, Kolber et al. (1998) noted a 15 to 20% higher *Fm* after 10 s dark-adaptation.



Plot of a single MT data sequence. The interval between flashlets during the relaxation phase started at 800 µs, but was increased by 20% between consecutive flashlets.

The inset shows the relaxation phase in more detail.

All of the examples shown above were collected from a mixed population of green algae, at a chlorophyll concentration of approximately 26 μg l^{-1} (Chl *a* in acetone equivalent). A series of 9 concatenated protocols was used to collect all of the above data in one go.

Reference: Kolber ZS, Prásîl O and Falkowski PG (1998) Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. *Biochim. Biophys. Acta* 1367: 88-106.