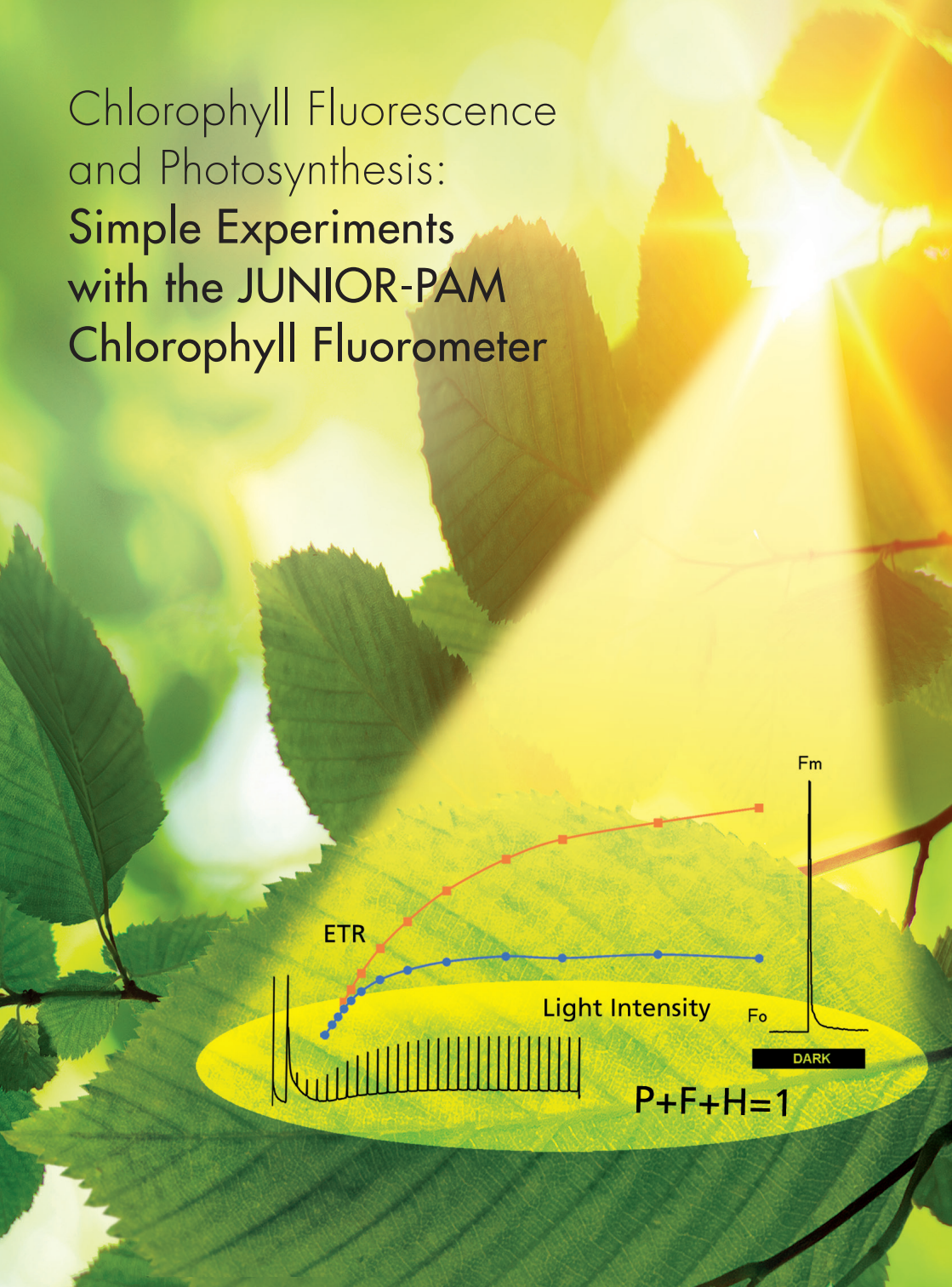
Chlorophyll Fluorescence and Photosynthesis: Simple Experiments with the JUNIOR-PAM Chlorophyll Fluorometer



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Chlorophyll Fluorescence and Photosynthesis: Simple Experiments with the JUNIOR-PAM Chlorophyll Fluorometer

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2.121 / 04.20
First Edition
SimpleExperiments01.docx

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Printed in Germany

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1 Preface

In 1997, Ulrich Schreiber published a booklet entitled: Chlorophyll fluorescence and photosynthetic energy conversion: 'Simple introductory experiments with the TEACHING-PAM Chlorophyll Fluorometer' of which he also produced a German version. It was a short tutorial clarifying important aspects that should be considered when making chlorophyll (Chl) *a* fluorescence measurements and illustrating several Chl *a* fluorescence phenomena in the form of short experiments. Given the number of times the two language versions have been cited it must have been a help to quite a few users of our instruments.

In the meantime (ca. 2008), the production of the TEACHING-PAM has been discontinued and it was replaced by the JUNIOR-PAM chlorophyll fluorometer. As there is still a need for a practical introduction to the use of our PAM chlorophyll fluorometers, we felt that it was a good idea to revise and update this booklet. Compared to its predecessor, the current booklet refers to the JUNIOR-PAM instead of the TEACHING-PAM. Some of the original experiments have been replaced.

The JUNIOR-PAM is the most basic fluorometer build by Heinz Walz GmbH and all the experiments described for this instrument can be made as well with most of the other Walz fluorometers. In a few experiments the suggestion is made to repeat the experiment(s) with e.g. green algae or cyanobacteria. Although there is no cuvette version of the JUNIOR-PAM, its fiber can be placed in a suspension, e.g. towards the top of a filled cuvette. Putting reflecting material (e.g. aluminum foil) on the outside walls of the cuvette, or using a sample holder with walls that reflect light, the signal quality can be improved.

We hope that our new version of the text will help the reader to become more familiar with our instruments and will allow him/her to use our instruments with confidence.

2 Introduction

The introduction consists of two parts. A text written by Todd Kana which focuses on the concept and the parameters that can be derived from fluorescence measurements using modulated measuring light and saturation pulses, and a second more mechanistic approach, which uses the physiological properties of the photosynthetic apparatus as a starting point for a discussion of fluorescence measurements written by Gert Schansker.

2.1 The concept of PAM fluorometry

2.1.1 PAM fluorometry and the detection of photosynthesis

PAM (Pulse Amplitude Modulation) fluorometry is a technique that provides a window into the workings of photosynthesis. It is almost magical in its capability. By shining a beam of light on a leaf and measuring the light re-emitted by the leaf, it is possible to measure not only the photosynthetic activity but also several underlying und regulatory mechanisms that control the photosynthetic process. PAM fluorometry measures the photosynthetic activity inside a leaf with little or no external influence. Just as a camera can take a color image of a leaf, an imaging PAM camera can take a picture of photosynthesis within the leaf. With a brief pulse of strong light (<1 second), the PAM image is ‘developed’ and a spatial picture of the rate of photosynthetic electron transport (photosynthesis) is produced. Whether it is with imaging or through a more common

spot/point measurement (only a few mm^2 of leaf is measured, which is the case that will be discussed here), the information obtained with the PAM technique has proven to be powerful for understanding mechanisms of photosynthesis and how photosynthesis responds to changes in the environmental conditions and stress. PAM fluorometry allows us to monitor the dynamic nature of photosynthesis.

This booklet is designed to introduce PAM fluorometry and provide a series of exercises and experiments to teach how this technique can be used to study the behavior of photosynthesis and the photosynthetic apparatus. In learning PAM fluorometry it helps to start with an understanding of some basic principles and to think in terms of *dynamic* processes with *feedback* effects. We all know that light drives photosynthesis. Sunny conditions are a stronger driving force of photosynthesis than shady conditions. However, it is more complicated than that. The *effectiveness* of the sunlight in driving photosynthesis must be considered and that is determined by the ability of the photosynthetic apparatus to handle the incoming light rays. Plants growing under deep shade conditions have adaptations that allow them to work very efficiently at low light intensities but become damaged when exposed to full sunlight. Plants regularly exposed to full sunlight are able of handling high light intensities but are often not very efficient at handling low light intensities. Plants make tradeoffs. If they have to acclimate to low light, they often invest in large PS II antennae and a low Calvin-Benson cycle capacity. If they have to acclimate to high light, they often invest in more PS II reaction centers with smaller antennae and high Calvin-Benson cycle capacities. Shifting a low light acclimated plant all of a sudden to high light will lead to the absorption of a lot of light energy the plant cannot use and will potentially lead to damage. Shifting a high light acclimated plant all of a sudden to

low light will lead to a low absorption of light energy and low photosynthetic activity.

Consider an analogy. On a sunny day more *photons* (light energy packages) reach your retina and your eye acts as a light meter registering bright light. Photosynthesis 'sees' light similarly, in that the light intensity (hereafter referred to as *irradiance*) is felt by the eyes of the photosynthetic apparatus (i.e. PS II and PS I). Your eyes can modify the amount of light reaching the retina through dilation or contraction of the pupils. Consider entering the outdoors under bright sunshine. The initial blinding effect (energy overload) is followed by adjustment and *acclimation*. The adjustment of vision happened through biological feedback mechanisms, and the sensation is altered. So too does the photosynthetic apparatus respond to such an energy overload of incoming irradiance through feedback mechanisms that lead to the dissipation of the excess light energy and modify the efficiency of light utilization based on the actual physiological state of the photosynthetic apparatus.

The concept of *physiological state* is important for the understanding of PAM fluorometry. The physiological state is generally related to environmental conditions experienced recently (memory effects), particularly those that impose stress, and how they are manifested physiologically. For instance, drought or high temperatures can cause changes in the photosynthetic apparatus that affect light utilization and photosynthesis rates and the physiological state may remain altered well after the stress was alleviated. If the stress caused damage to the photosynthetic apparatus, it is analogous to a wound that needs time to heal. Even less persistent stresses in the environment, such as rapid changes in irradiance on a cloudy day or flickering light in forests related to branches and trees moving in the wind (sunspots), can have

measurable effects on the efficiency of light utilization. Dynamic changes in the environment happen on many different time scales and in response to many environmental variables. The plant senses these changes. E.g., PS II and the cyt b6f release more protons in the lumen than ATP-synthase can use, indicating that the Calvin-Benson cycle activity cannot keep up with electron transport activity. The lumen becomes more acid and this causes a slowdown of electron flow towards PS I and induces a mechanism that increases heat emission by the PS II antenna. The lumen pH is part of a feedback mechanism by which the photosynthetic apparatus brings electron transport and Calvin-Benson activity in balance. These changes can be measured and monitored by a PAM fluorometer.

2.1.2 Light harvesting

A fundamental parameter that can be measured with a PAM fluorometer is the conversion efficiency of light energy into chemical energy (i.e. photosynthesis) in photosystem II. This efficiency is commonly called *Yield* in PAM nomenclature, a shortening of ‘quantum yield’. Yield is an important concept in photochemistry, and it relates to the probability that an absorbed photon (a quantum) initiates a chemical reaction, which in the case of photosynthesis involves the transfer of an electron from a donor molecule to an acceptor molecule. Thus, we think of one absorbed photon driving a charge separation between an electron donor and an electron acceptor increasing the redox energy of that electron; converting excitation/light energy into redox energy (chemical energy). A charge separation is not stable, and trapping is achieved by transferring the electron away from the charge separation site. A Yield measurement of 0.6, for example, means that 60% of the

absorbed photons successfully carry out a photochemical or photosynthetic reaction. Once a photosystem has trapped a package of excitation energy, it needs some time before it can trap the next energy package. If it is ready for trapping, the Yield is ~0.83; if it is not ready, the Yield is 0. The regeneration time is for example dependent on the speed with which the trapped electron can be moved forward into the electron transport chain. This is the main reason why in the light, for an unstressed leaf, the Yield can range between 0 and ~0.83.

The fate of absorbed light. Measurements carried out with a PAM fluorometer consist of three components: measuring light, actinic light and saturation pulses. With the actinic light photosynthetic activity is induced and the saturation pulses are applied to determine the maximum fluorescence yield. The third component, the modulated measuring light, induces by itself nearly no photosynthesis, but measures/monitors variations in (the fluorescence) Yield. Only the fluorescence response elicited by the modulated light is registered by the fluorometer. The instrument is blind to the continuous actinic light and the saturation pulses.

In the case of photosynthesis, fluorescence is the *emission* of light by chlorophyll *a* (a colored and therefore light absorbing molecule). When a photon of a suitable energy (wavelength) is absorbed by a pigment such as chlorophyll, the energy level of the molecule is raised, which means in practice that an electron bound to that molecule is brought into a higher orbit. The pigment molecule is said to be in an *excited state*. Excited states are generally unstable, lasting only 10^{-12} to 10^{-9} seconds after which they lose the absorbed energy and revert to the original *ground state*. Central to PAM fluorometry is the fact that an excited state can have three alternative fates. The energy of the excited state can be 1)

emitted as a photon (fluorescence), 2) lost as heat, or 3) transferred to another chlorophyll molecule/trapped as chemical energy (photochemistry). For photosynthesis, the relative proportion of each of these three fates is variable, but their sum always adds up to 1. Thus, if one pathway increases, there must be a decrease in one or both of the other two pathways. The cleverness of the *Quenching Analysis* that was developed for PAM fluorometers is that it measures a single entity, fluorescence, to determine changes in both heat and photochemistry associated with light absorption. Whereas photochemistry equates to photosynthesis, heat equates to energy *dissipation* or loss. Heat dissipation turns out to be an important pathway regulated by the photosynthetic apparatus to protect itself from damage by excess light. How can fluorescence measurements determine both heat and photochemistry pathways? This requires an understanding of the photosynthetic process.

In leaves, light is absorbed by chlorophyll and carotenoid pigments bound to proteins. The proteins control the orientation of the pigments relative to each other on a nanoscale, assuring efficient energy transfer between these pigments. The protein environment may also modify the absorption properties of the bound pigments. These pigment-protein complexes serve as *antennae* absorbing light falling on the leaf. Within a single chloroplast, there can be thousands of antenna complexes and a single antenna complex binds typically hundreds of chlorophyll and carotenoid molecules. Photons are absorbed by single chlorophyll or carotenoid molecules, but the induced excited states of that molecule can very quickly and very efficiently transfer this excitation energy to the surrounding pigments within the antenna complex and to a limited extent to the antennae of other *photosystems*. The excited state

energy being rapidly and randomly transferred throughout the antenna will at a certain point reach a complex of four chlorophyll molecules (a dimer and two accessory pigments) in the *reaction center* of the photosystem. Here, the excitation energy is used to drive a charge separation (an electron transfer) between one of the accessory pigments (the electron donor) and an electron *acceptor*. This is the beginning of the photosynthesis process.

Plant photosynthesis utilizes two types of antennae and reaction centers, referred to as *photosystems I and II*. Of the two photosystems only photosystem II (PSII) possesses the characteristics useful for PAM fluorometry allowing a fluorometric measurement of photosynthesis. Our discussion focuses on PS II only. The physiological state of PS II determines the lifetime of the excitation energy. Is it short, the fluorescence yield is 1-2%; is it long the fluorescence yield will go in the direction of 10%. It is this ~5-6-fold increase from F_0 to F_M, the *variable fluorescence*, that provides information on the alternative energy pathways, heat and photochemistry. Variable fluorescence is a key concept and is only observed in photosynthetic systems. By contrast, extracted chlorophyll in acetone, which is used as a common fluorometric assay for the chlorophyll concentration, exhibits a constant fluorescence probability or yield. There are no factors, like the redox state of Q_A, that modulate this probability: fluorescence intensity is proportional to chlorophyll concentration at constant illumination and proportional to irradiance at constant chlorophyll concentration. In the case of photosynthesis on the other hand, the lifetime of excitation energy is variable; it depends on the degree of heat dissipation and photochemistry, which are under biological regulation.

2.1.3 Stable charge separations

Open and closed photosystems. Towards understanding the basis of variable fluorescence, we begin with the concept of *open* and *closed* photosystems, one of the central concepts of PAM fluorometry. Consider a leaf in darkness. None of the photosystems are photo-energized and all of them can conduct photochemistry if a photon is absorbed. We consider that the photosystems are open (for doing the business of photochemistry). Open photosystems are in a low fluorescence state. The alternative state is a reaction center that has just undergone a charge separation and the photosystem is not yet ready for the next charge separation. During this time interval, the photosystem is considered to be *closed* (no additional photochemistry possible). In a closed photosystem, the probability that the excited state will be dissipated as heat or fluorescence increases enormously. Closed photosystems are in a high fluorescence state.

How long are photosystems closed? A central activity of PS II is a so-called stable charge separation. That is an electron transfer from the four reaction center chlorophylls (often called P680) on the donor side of PS II and the second electron acceptor (a quinone called Q_A) on the acceptor side of PS II. Each stable charge separation requires a re-reduction of $P680^+$ and a re-oxidation of Q_A. In the presence of sufficient substrate (oxidized plastoquinone (PQ) pool) this regeneration time can take up to 2 ms. However, under many light conditions the real rate limiting step is further downstream (the re-oxidation of reduced PQ molecules by the cytochrome b6f complex located between the two photosystems). In the light, a turnover time of 1 electron per 5 ms has been determined.

We can now consider how a population of photosystems within chloroplasts, cells, and leaves behave over a range of irradiances. For this discussion we will refer to the fluorescence yield (the probability that excitation energy is emitted as fluorescence). The way the fluorescence yield is measured with a PAM fluorometer is described below. One of the standard experiments found in the software of all PAM instruments is the light curve. In our thought experiment, we make use of the light curve concept: we slowly increase irradiance from darkness to a high irradiance. Under very dim light, the rate of photon absorption by any given photosystem is much lower than the ms time it takes to photochemically process the energy (in practice the time to re-oxidize Q_A). Therefore, almost all photosystems are in the open state whenever a photon is absorbed. This is the condition where photochemistry is the preferred fate of the excited state and fluorescence yield is minimal.

As we raise the irradiance on the leaf, the excitation rate of the population of photosystems increases and total photochemistry (i.e. photosynthesis) increases proportionally, as long as the interval between excitations is longer than the ms limiting rate for reopening a closed photosystem. As we raise the irradiance further, there will be some point, when a few photosystems absorb a photon while they are in the closed state. In that case the excitation energy has to be dissipated as fluorescence or heat since photochemistry is not an option, and the fluorescence yield (as well as the yield of heat dissipation) of those (closed) photosystems is high. At the level of a leaf and the whole population of photosystems, the measured fluorescence yield is the sum of the fluorescence emanating from the large population of open photosystems and small population of closed photosystems. The integrated fluorescence yield exhibits a small increase from its minimum (F_0). By extension, a further increase in irradiance leads to a greater

proportion of photosystems that absorb a photon while in the closed state and consequently the fluorescence yield increases proportionally. At the limit, where there is sufficient irradiance to close all of the photosystems, the measured fluorescence yield becomes maximal. This limit is referred to as *saturating* irradiance and in practice, leaves require an irradiance ~3-5 times greater than full sunlight or 6000-10000 µmol photons m^{-2} s^{-1} to close all photosystems and maximize the fluorescence yield. Experimentally, this high fluorescence yield can be measured with a brief pulse (~0.5 s) of high intensity light. We can now introduce some standard terminology. For a darkened leaf with all open photosystems, the fluorescence signal induced by very low intensity PAM measuring light is termed F_0. For a leaf exposed to a *saturating pulse* of light, the fluorescence yield is termed F_M.

2.1.4 Quantification of fluorescence

Fundamental quantum yield of photosynthesis. The preceding discussion describes two limits. Darkness is needed for the condition of 100% open photosystems and minimum fluorescence yield (F_0) and saturating irradiance for the condition of 0% open photosystems and maximum fluorescence yield (F_M). Because we are interested in photosynthesis, we can say, alternatively, that the condition of darkness corresponds to the maximum (potential) photochemical yield, whereas saturating irradiance with all photosystems closed corresponds to zero photochemical yield. A commonly used term for describing the effect of photochemistry on fluorescence yield is '*photochemical quenching*', i.e. photochemistry quenches (prevents/reduces) fluorescence emission by excited states. Thus, an increase in fluorescence signal from a PAM fluorometer would correspond to a decrease in photochemical yield for a population of photosystems. The limits, F_0 and F_M, can be

used to evaluate the relative excitation energy going to the photochemistry pathway. The difference between the F_M and F_0 levels (F_M-F_0) corresponds to the maximum difference in photochemical quenching ability of the photosystems II.

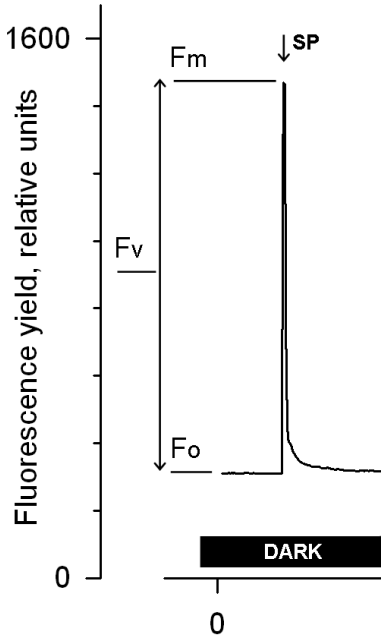

Fig. 2-1: SP analysis of a dark acclimated leaf.

On turning on the measuring light, the F_0 level is induced. This is a reflection of the excitation energy that is not used for photochemistry in open reaction centers. With a saturation pulse all reaction centers are closed (photochemistry = 0) and the F_M level is induced. This is a reflection of the excitation energy lost as fluorescence when photochemistry is 0 and where it is assumed that probability that excitation energy is lost as heat remains unchanged: fluorescence and heat change proportionally. Photochemical yield is now defined as the increase of the fluorescence yield on closing all reaction centers (F_V) divided by the total fluorescence yield when all reaction centers are closed (F_M): F_V/F_M.

To express this as photochemical Yield, which is the fraction of total energy going to photochemistry, F_M-F_0 must be normalized to the condition of maximum fluorescence, F_M, which is a measure of total fluorescence quenching. I.e. (F_M-F_0)/F_M (see Fig. 2-1). The numerator corresponds to the extent of photochemical quenching of the maximum fluorescence yield, F_M. This equation is commonly abbreviated as F_V/F_M, where F_V is *variable fluorescence*. F_V/F_M is

the fluorescence expression for photochemical Yield of PSII and it is a measure for the maximum quantum efficiency of photosynthesis when all PS II reaction centers are open and in the absence of physiological mechanisms that can potentially down-regulate Yield. It is typically measured in the dark-acclimated state of photosynthesis.

F_V/F_M is typically near 0.8 for healthy leaves, indicating that 80% of the absorbed energy is converted to photochemical energy (see section 2.2.4 and experiments 4.1.5, 4.1.6 and 4.1.7 for a discussion of the effect of PS I fluorescence on this value). The remaining 20% of energy is not utilized for photochemistry and is dissipated as fluorescence or heat. This 20% energy dissipation yield is considered to be non-regulated. It is equal to F_0/F_M and termed Y(NO).

Influence of regulated heat dissipation. The photosynthetic apparatus is capable of dissipating additional energy as heat by increasing the probability that the energy is lost by this pathway. This process we can monitor because it reduces the fluorescence yield. This is an evolutionary strategy, which reduces the lifetime of excitation energy in the antenna of closed reaction centers and thereby protects the photosynthetic apparatus against the formation of oxygen radicals. In plants where this also leads to fluorescence quenching in open reaction centers (F_0 quenching), it will also result in a down-regulation of PS II activity. To assess this, we need to compare the fluorescence response of PS IIs with an upregulated heat pathway to that under dark-acclimated conditions where the heat pathway has the lowest probability. The dark acclimated state is our reference state for a system that does not express regulated heat dissipation and F_M and F_0 correspond to that condition. An increase in the heat pathway probability occurs

at the expense of the fluorescence pathway (F_M quenching) and also at the expense of the photochemistry pathway in the case of F_0 quenching. So, we expect to observe a decline in F_M and possibly F_0. For this discussion, we will focus on F_M, since this is what is commonly done in the literature. A saturation pulse measurement for a leaf that has up-regulated light-induced heat dissipation will result in an F_M signal that is lower than that of a dark-acclimated leaf. These two conditions are distinguished by using *F_M'* for the illuminated condition (vs F_M for the dark state). Thus, F_M' is lower than F_M because it includes fluorescence quenching associated with a regulated heat pathway. It should be noted here that there are more processes that can lower F_M and the F_M decrease associated with the up-regulated heat pathway relaxes within approx. 200 s of darkness and can in that way be distinguished from other processes lowering F_M (see e.g. the experiment of 4.1.12, where a state transition experiment is described; state transitions also modify F_M by changing the PS II antenna size).

Photosynthetic yield under illuminated conditions. The special case of the dark-acclimated F_V/F_M does not consider the effect of background irradiance on photosynthesis. Photochemical Yield in the light is the key measurement for determining photosynthesis using fluorometry. In the history of PAM fluorometry, a key conceptual framework was presented by Genty et al. (1989), wherein the authors proposed that the operative photosynthetic yield in the light was the product of the fundamental yield of the PS IIs (assumes that PS II is not down regulated by light) multiplied by the fraction of photosystems that are open. That is, only open PS IIs are relevant to photosynthesis and those photosystems operate at some 'fundamental' efficiency for photochemistry. Thus, for a leaf,

if photosystem efficiency (F_V/F_M) is 0.8 and only half of the photosystems are in the open state, then the leaf exhibits a photosynthetic efficiency of 0.8 x 0.5 = 0.4.

How do we measure the fraction of open photosystems? For that, we return to the concept of F_0 and F_M (or F_0' and F_M') representing the extremes of 100% open and 0% open, respectively. As the actinic irradiance is increased from darkness, the fraction of open photosystems II declines and F increases toward F_M in proportion to the fraction of closed reaction centers. Thus, the intensity of F (in light) relative to F_0' and F_M' is expressed as:

$$q_P = \frac{F'_M - F}{F'_M - F'_0} \qquad \text{Eq. 1}$$

where q_P is the fraction of open photosystems and referred to as *photochemical quenching*.

In accordance with the concept by Genty et al., operational photosynthetic efficiency is given by the maximum PS II efficiency in the light times q_P:

$$Y(II) = \frac{F'_M - F'_0}{F'_M} \cdot q_P = \frac{F'_M - F}{F'_M} \qquad \text{Eq. 2}$$

Y(II) is the PSII operating efficiency or effective photochemical quantum yield of PS II.

Significantly, F_M' and F are measured by a PAM fluorometer in the light, so no dark acclimation measurement is needed and Y(II) is based on a simple saturation pulse measurement requiring <1 sec.

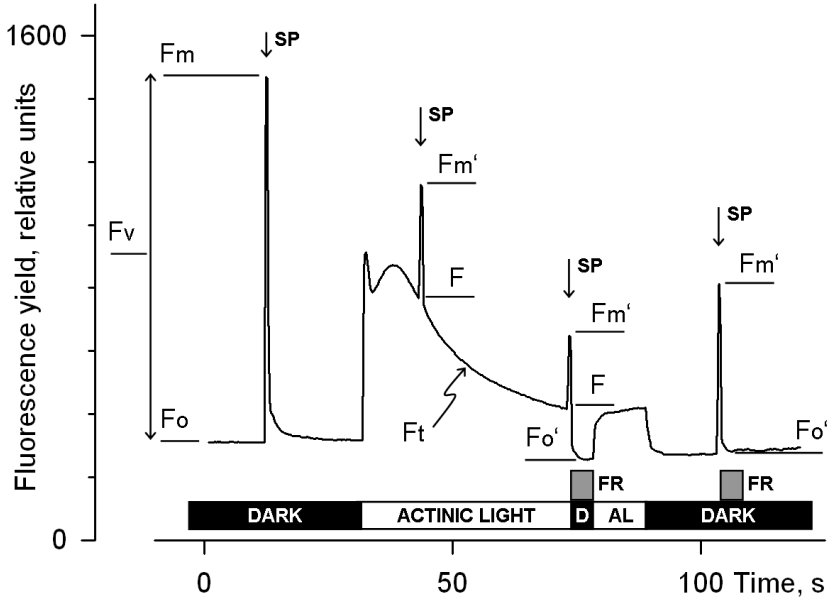

Fig. 2-2: Induction experiment.

The cardinal points relevant to a Quenching Analysis, which can be determined on the basis of an induction experiment.

Converting Y(II) to photosynthesis. Y(II) is the utilization efficiency of absorbed photons for photosynthesis. To convert Y(II) to photosynthetic rates, we require an independent measurement of absorbed light. For photosynthesis, irradiance is typically measured in units of photon flux rather than Watts, another commonly used unit of light energy. A photon basis has advantages because high energy blue photons are just as efficient in inducing photochemical electron transfer as low energy red photons. Photosynthesis is color-blind, i.e., once a photon is absorbed and turned into excitation energy it can induce a charge separation. We measure the photon flux in units of µmol photons m^{-2} s^{-1} within the *photosynthetically active radiation* (PAR) spectrum of 400-700 nm.

The S.I. symbol for irradiance is E (Einstein), which is the *incident* irradiance on the surface of a leaf and can be determined with a light meter. Green leaves are green because they absorb green light inefficiently, so photon absorption is <100% of incident light. For typical leaves, ~85% of the incident photons are absorbed. This fraction is referred to as *absorptance* (A). Finally, to convert photon absorption and photochemical efficiency to photosynthesis, we have to account for the fact that one electron requires two photochemical steps: PSII and PSI. In leaves the ratio between the two photosystems is often assumed to be 1.

Given these considerations, we can express a photosynthetic rate as an '*electron transport rate'* (ETR) from the following equation:

$$ETR = Y(II) \cdot E \cdot A \cdot 0.5 \qquad \text{Eq. 3}$$

For PAM measurements, E is measured with a PAR sensor at the level and orientation of the leaf. For liquid samples, for example in a cuvette, special spherical PAR sensors are used. A is estimated or measured as the incident light - (transmission of PAR across the thickness of the leaf + the reflected light), and Y(II) is determined on the basis of a saturation pulse measurement (Fig. 2-2). ETR can be scaled to O_2 equivalent rates by dividing by 4 (4 e^- /O_2).

Regulated heat dissipation and Non-Photochemical Quenching. Plants have evolved a number of mechanisms to regulate light harvesting and the heat dissipation pathway of photosynthesis. Photosynthetic organisms have found ways to make efficiently use of low light intensities while at the same time efficiently dissipating excess absorbed light that cannot be used for photochemistry. Excited chlorophyll states are potentially harmful to plants. The probability is not very high, but excitation energy can lead to

the formation of singlet oxygen radicals that can cause oxidative damage to PS II and the photosynthetic apparatus. Upregulating the 'safe' heat dissipation pathway under high light conditions can strongly reduce this risk.

As explained above, upregulation of the heat dissipation pathway means less fluorescence and potentially less photochemistry, which can be detected as a decrease in F_M and F_0. It is one of several processes that affect the F_M level. Other processes are state transitions (which modify the PS II antenna size), chloroplast movements and photoinhibition. Together, these processes are called, in a manner similar to photochemical quenching described above, *non-photochemical quenching (NPQ)*. How can we quantify NPQ and how can we separate the different NPQ-components?

To start with the last question first, the different NPQ components can be separated on the basis of their relaxation kinetics following a light-to-dark-transition. Recovery from photoinhibition takes hours, for example. State transitions can also be reversed (and quantified) by far-red light (see 4.1.12 and 6.4).

In the PAM fluorometry literature, two main mathematical descriptions of non-photochemical quenching are used. Originally, non-photochemical quenching was defined as a fractional loss of variable fluorescence.

$$q_N = 1 - \frac{F'_V}{F_V} = 1 - \frac{F'_M - F'_0}{F_M - F_0} \qquad \text{Eq. 4}$$

However, this means that non-photochemical quenching is by definition a value between 0 and 1. In order to get a broader range of values, a second approach was developed, which assumed that non-photochemical quenching could be described by Stern-

Volmer kinetics. The Stern-Volmer concept is based on the idea that a collision between an excited state (for example of a chlorophyll molecule) and a quencher (for example a carotenoid) accelerates the de-excitation of that excited state. Within the Stern-Volmer concept NPQ is proportional to the number of quenching centers and can be described by:

$$NPQ = \frac{F_M}{F'_M} - 1 \qquad \text{Eq. 5}$$

It has been argued that PS II does not fulfill the requirements of a system that can be described by Stern-Volmer kinetics (see e.g. Holzwarth et al. 2013), however, the parameter NPQ was shown experimentally to correlate linearly with e.g. the extent of xanthophyll epoxidation, which is one of the determinants of non-photochemical quenching (Bilger and Björkman 1991, Gilmore and Yamamoto 1993).

A bit confusing is that NPQ is often used both for the concept of non-photochemical quenching and for the parameter calculated on the basis of Stern-Volmer kinetics defined above.

More recently, it has started to become popular to define non-photochemical quenching as a yield: Y(NPQ). That means, as discussed above:

$$Y(II) + Y(NO) + Y(NPQ) = 1 \qquad \text{Eq. 6}$$

The derivation of Y(NO) and Y(NPQ) will not be discussed here, just the equations are given. For a more in-depth discussion of these equations see Klughammer and Schreiber (2008).

$$Y(NO) = \frac{F}{F_M} \qquad \text{Eq. 7}$$

and:

$$Y(NPQ) = Y(NO) \cdot NPQ = \frac{F}{F'_M} - \frac{F}{F_M} \qquad \text{Eq. 8}$$

F_M is our reference signal for zero NPQ and a decline observed in an F_M' measurement must correspond to an increase in NPQ.

Equations 7 and 8 given above, require the determination of F_M, the maximum fluorescence yield determined under dark acclimated conditions, which means no non-photochemical quenching and all PS II reaction centers open. The parameters discussed above are summarized in Table 1.

Mechanisms and time scales of NPQ. NPQ reflects a fluorescence yield change and such changes are in most cases (NPQ related to photoinhibition is an exception) due to modifications of the PSII antenna (core antenna + LHCII in plants) affecting photon absorption and the lifetime of the excited states. It is regulated by the pH difference that forms across the thylakoid membranes during photosynthesis. Formation of the delta pH across the thylakoid membranes is needed for ATP synthesis. Lumen acidification leads to protonation of a protein called *psbS* and activation of the enzyme VDE (*violaxanthin deepoxidase*) that catalyzes the formation of the carotenoids antheraxanthin and zeaxanthin out of violaxanthin (*xanthophyll cycle*). The complex interaction between protonated psbS, antheraxanthin/zeaxanthin and the light harvesting complexes of PS II (LHCIIs) causes upregulation of the heat dissipation pathway, which is detected as non-photochemical quenching.

During a dark-to-light transition several processes are induced in parallel and their kinetic effects on the fluorescence signal overlap. This means that it is difficult to separate the different processes. On a subsequent light-to-dark transition each of these processes

relaxes with its own kinetics. Deconvolution of these relaxation kinetics allows, in principle, a quantification of the contributions of the different processes to NPQ. These relaxation kinetics can be probed and monitored using saturation pulses. PAM instruments use increasingly longer time intervals between saturation pulses to allow on the one hand a resolution of the fast relaxation processes and on the other hand to avoid an effect of saturation pulses on the relaxation kinetics.

Table 1: Fluorescence Quotients.

Source	Equation
Maximum photochemical quantum yield of PS II (Kitajima and Butler, 1975)	$\frac{F_V}{F_M} = \frac{F_M - F_0}{F_M}$
Effective photochemical quantum yield of PS II (Genty *et al.*, 1989)	$Y(II) = \frac{F'_M - F}{F'_M}$
Coefficient of photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_P = \frac{F'_M - F}{F'_M - F'_0}$
Coefficient of photochemical fluorescence quenching assuming interconnected PS II antennae (Kramer *et al.* 2004)	$q_L = q_P \cdot \frac{F'_0}{F}$
Coefficient of non-photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_N = 1 - \frac{F'_M - F'_0}{F_M - F_0}$
Stern-Volmer type non-photochemical fluorescence quenching (Bilger and Björkman, 1990)	$NPQ = \frac{F_M}{F'_M} - 1$
Quantum yield of non-regulated heat dissipation and fluorescence emission: this quenching type does not require the presence of a trans-thylakoid ΔpH and zeaxanthin (Genty *et al*. 1996)*	$Y(NO) = \frac{F}{F_M}$
Quantum yield of light-induced non-photochemical fluorescence quenching (Genty *et al*. 1996)*	$Y(NPQ) = \frac{F}{F'_M} - \frac{F}{F_M}$

*Kramer *et al.* (2004) have derived more complex equations for Y(NO) and Y(NPQ). Klughammer and Schreiber (2008) have demonstrated that the equations by Kramer *et al.* (2004) can be transformed into the simple equations of (Genty *et al*. 1996) which are used by the PamWin-3 software.

2.2 Physiology of photosynthesis and fluorescence

2.2.1 Chloroplasts and the photosynthetic apparatus

The most common color of leaves is green. The blue and red wavelengths of visible light are absorbed efficiently by the chlorophyll and other pigment molecules in the leaf and only green light is reflected or transmitted to some extent. Looking at leaf cuts under the microscope, the green color is concentrated in small organelles in the cells. These organelles are called chloroplasts, where 'chloro' is derived from the classical Greek word for pale green.

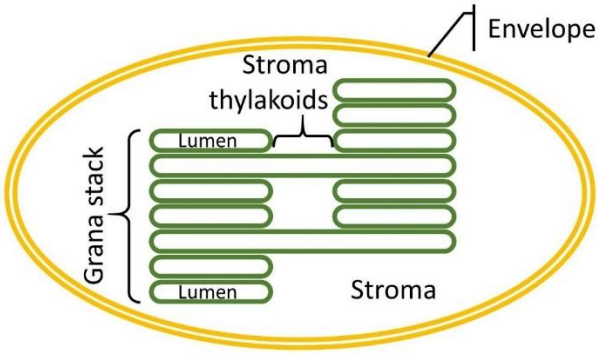

Fig. 2-3: Very schematic representation of a chloroplast and its main structures.

Zooming in on the chloroplasts, stacks/piles of membranes are found. These membranes are called thylakoid membranes and the stacks of thylakoid membranes are called grana. Thylakoid membranes form a kind of vesicles with an inner area that is called lumen and an outside area that is called stroma (indicated in Fig. 2-3 and Fig. 2-4). In the thylakoid membranes four large protein

complexes are found: photosystem II (PS II; Fig. 2-4 and Fig. 2-5), the cytochrome b6f complex, photosystem I (PS I) and ATP synthase (Fig. 2-4). These four protein complexes and the molecules acting as mobile electron carriers between them (plastoquinone molecules, plastocyanin, ferredoxin) are responsible for the conversion of light energy (photons) into chemical energy (ATP and NADPH). This can be considered the biophysical part of the photosynthetic process.

Photosynthesis is a central process in plants and photosynthetic organisms. Photosynthesis is the energy source for photosynthetic organisms and, indirectly, also the energy source for nearly all other organisms on Earth. Oxygenic photosynthetic organisms use water as electron and proton source. These organisms split two molecules of water into 4 electrons, 4 protons and a molecule of oxygen. These 4 electrons drive electron flow through an electron transport chain leading in the end to the reduction of $NADP^+$. The protons contribute to the pH difference over the photosynthetic membrane that drives ATP synthase and results in the synthesis of ATP out of ADP+P. The oxygen molecules are mainly liberated into the air and over time have created an oxygen enriched Earth atmosphere. Without it, our existence on Earth would be impossible. The oxygen produced by oxygenic photosynthesis has also led to the formation of the ozone layer, which protects us against cosmic UV-C radiation and keeps DNA mutation rates in check.

The reaction center of PS II is formed by two protein subunits: D1 and D2 that carry all the co-factors needed for charge separations and charge stabilizations. The P680 indicated in Fig. 2-3 stands for a complex of four Chl molecules, a dimer and two accessory Chls. Excitation energy reaching the PS II reaction center induces

a charge separation between the accessory Chl bound to the D1 protein and Pheophytin (Pheo). This is a very unstable state and the recombination reaction has a relatively high probability, after which the excitation energy is returned to the antenna. The excitation energy can, however, be transferred again to the reaction center and induce again a charge separation. This is called the reversible radical pair mechanism (van Grondelle 1985, Schatz et al. 1987, 1988).

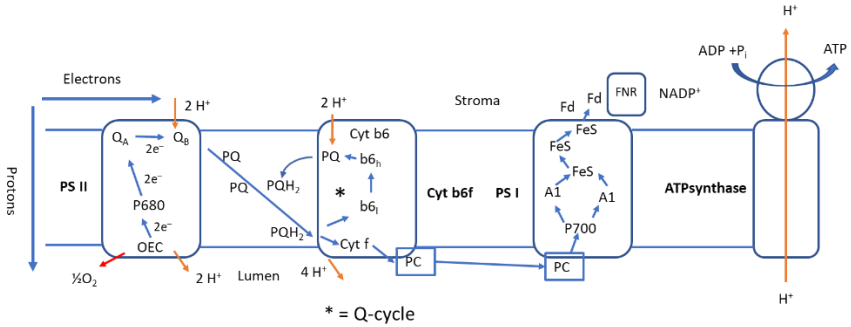

Fig. 2-4: Schematic representation of the photosynthetic electron transport chain.

Linear electron transport and Q-cycle are indicated (blue arrows), as well as proton uptake and release (orange arrows) and oxygen release (red arrow). The proton to electron ratio can be further increased when cyclic electron transport around PS I and chlo-rorespiration (not indicated) would occur. A1 = a phylloquinone (comparable with Q_A in PS II); $b6_h$ and $b6_l$ are the high and low potential hemes of cytochrome b6; Fd = ferredoxin; FeS = iron sulfur cluster; FNR = ferredoxin-NADP$^+$-reductase; OEC = oxygen evolving complex; P680 and P700 = reaction center Chl complexes of PS II and PS I respectively; PC = plastocyanin; PQ and PQH_2 = plastoquinone and plastoquinol (reduced and protonated PQ), respectively; Q_A and Q_B = first and second quinone electron acceptor of PS II, respectively.

The charge separation can be stabilized by transfer of the electron to Q_A and subsequently to Q_B; at the same time the electron hole can be transferred first to a tyrosine of the D1 protein subunit (Tyr_Z) and from there to the manganese cluster (indicated as oxygen evolving complex in Fig. 2-7). The redox states of the Mn-cluster are called S-states, where S1 represents the redox state in the dark-adapted state and S2 the state after one charge separation. The state $S_2Q_B^-$ is 300,000 times more stable than the state $P680^+Q_A^-$. The electron transfer described here is indicated by blue arrows in Fig. 2-5.

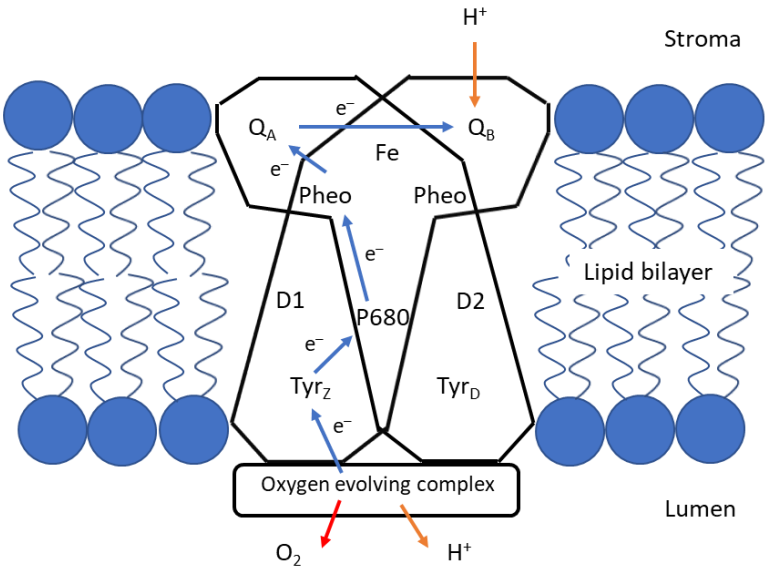

Fig. 2-5: Schematic representation of the reaction center of PS II.

The electron transfer pathway is indicated (blue arrows), as well as proton uptake and release (orange arrows) and oxygen re-lease (red arrow).

In summary, the three most important functions of PS II are: 1. Light harvesting; 2. Charge separation and charge stabilization; 3. Water splitting, yielding electrons (for the reduction NADP+) and protons (to drive ATP synthesis).

Experimental probes

The ultimate goal of all biological research is to understand organisms *in vivo*. To study organisms under *in vivo* conditions we need non-invasive techniques. For photosynthesis research quite a few non-invasive techniques are available of which Chl *a* fluorescence is an important one.

We can study the emission and uptake of oxygen, the uptake and release of CO_2, the evaporation of water, leaf temperature, the redox states of plastocyanin, P700 and ferredoxin, Chl *a* fluorescence, delayed fluorescence, etc. In the literature, the photosynthetic apparatus is often split in light reactions, i.e. reactions related to the photosynthetic electron transport chain (Fig. 2-4) and dark reactions, i.e. the Calvin-Benson cycle and associated processes.

Being a central process makes photosynthesis an attractive probe for monitoring the state of a plant or photosynthetic organism in general. If the photosynthetic apparatus is stressed, we may assume that the organism as a whole is stressed. And of the techniques mentioned above, Chl *a* fluorescence is the most easily measured and also the most versatile.

2.2.2 Chlorophyll fluorescence

Chl *a* fluorescence is emitted by all photosynthetic organisms, which harvest light with the help of chlorophyll molecules. That the fluorescence intensity has particular kinetics following a dark-to-light transition was first observed in 1931 by Kautsky and Hirsch and this induction phenomenon we still study and use almost 90 years later. In contrast to Chl *a*, Chl *b* does not emit fluorescence, because it irreversibly transfers excitation energy on a fs (femtosecond) time scale to Chl *a*.

Light energy absorbed by the antennae of PS II and PS I is transferred randomly from Chl *a* to Chl *a*. Per unit of time there is a certain probability that it is emitted as Chl *a* fluorescence. This means that fluorescence emission is a function of the time the excitation energy is moving from one Chl *a* molecule to the next. Depending on the redox state of PS II and the photosynthetic electron transport chain somewhere between 2 and 10% of the absorbed light is re-emitted as Chl *a* fluorescence. It is a minor process of limited physiological relevance. Even so, many articles about Chl *a* fluorescence have been published, because it reflects photosynthetic activity and, in addition, can be used to study changes in heat emission and, thereby, regulatory mechanisms affecting photosynthesis.

In essence three things can happen to absorbed light: 1. It can provide the energy for a charge separation and thereby induce photosynthesis (P); 2. It can be emitted as heat (H); 3. It can be emitted as Chl *a* fluorescence (F), where

$$P + H + F = 1 \qquad \text{Eq. 9}$$

Heat emission is one of the processes responsible for non-photochemical excitation-energy-quenching (discussed in more detail below).

With a short pulse of saturating light (discussed in detail below) we can transiently close all PS II reaction centers. In that case P = 0 and the formula is H + F [= F_M] = 1. It is assumed that a saturation pulse does not affect the rate constants for heat emission and Chl *a* fluorescence and this means that P going to 0 is accompanied by a proportional increase of H and F.

A saturation pulse by itself does not change the rate constant for heat emission. However, there are quite a few other treatments that do affect this parameter. For example, illuminating a dark-adapted leaf leads to the transformation of the xanthophyll violaxanthin into zeaxanthin. The presence of zeaxanthin increases the probability that excitation energy is lost as heat (it increases the rate constant for heat emission) and this is reflected by a lower fluorescence level (when P = 0): instead of F_M a lower F_M' value is measured. Using saturation pulses, Chl *a* fluorescence can be used to probe both photochemical quenching (reflecting the redox state of the photosynthetic electron transport chain) and non-photochemical quenching (e.g. caused by an increased rate constant for heat emission).

2.2.3 Time Domains

Chl *a* fluorescence is special, because the time domain measured determines the processes Chl *a* fluorescence probes (Fig. 2-6): In the sub-microsecond time domain mainly PS II antenna related processes are probed. In the femtosecond time range this can for example be the energy transfer between Chl *b* and Chl *a* and in

the picosecond time domain it is possible to obtain information on energy equilibration within the PS II antenna.

At times longer than 1 µs Chl *a* fluorescence kinetics start to reflect the electron transport reactions in the photosynthetic electron transport chain. First, only reactions inside PS II (few ms), subsequently the whole electron transport chain (up to 200-1000 ms), and at longer times (minutes or longer), the balance between photosynthetic electron transport chain and Calvin-Benson cycle activity (steady state). With special light protocols it is possible to focus on specific reactions.

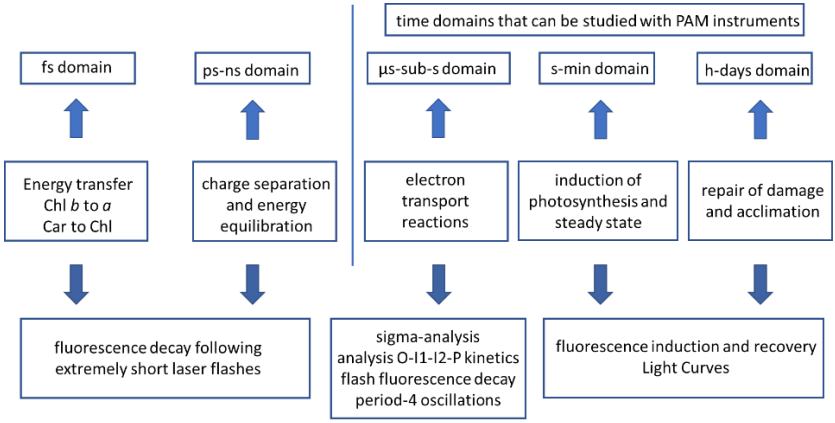

Fig. 2-6: Overview of the time domains that can be studied on the basis of Chl a fluorescence measurements.

Only the three slower time domains can be studied with PAM instruments.

However, it is only possible to interpret Chl *a* fluorescence signals in such detail because we know already a lot about these reactions. And other non-invasive techniques (near-infrared (NIR)

measurements probing PS I, delayed fluorescence, electrochromic shift measurements) are available to confirm the correctness of the interpretation of the fluorescence data.

2.2.4 F_0

On turning on the light, the Chl *a* fluorescence intensity increases almost instantaneously to a level called the F_0 level. This is the fluorescence intensity measured when PS II is still in the oxidized state (the PS II reaction centers are still open, that is, Q_A is in the oxidized (which is for quinones the neutral) state). In the subsequent tens of µs/ms the fluorescence intensity increases in parallel to a reduction of Q_A (that is the formation of Q_A^-) and the electron transport chain. That F_0 is larger than 0 when all reaction centers are open, indicates losses of excitation energy and a photochemical yield smaller than 1. In C3 plants F_0 consists of 25-30% PS I fluorescence emission and 70-75% PS II fluorescence emission. The excitation energy of an absorbed photon is randomly transferred from one chlorophyll molecule in the antenna to the next (Fig. 2-7) and even charge separations are reversible. The probability is very low, but on each transfer step the energy can be lost as either fluorescence or heat and as the lifetime of the excitation energy increases, accumulated energy losses as fluorescence (and heat) will increase as well. In C3 plants the PS II to PS I ratio is approximately 1. That the energy losses as fluorescence of PS II are much higher than those observed for PS I, is due to the fact PS II is a relatively slow reaction center (due to the reversibility of charge separations), whereas PS I is a faster reaction center (due to the near irreversibility of the charge separation; see discussions in Croce and van Amerongen 2011, 2013).

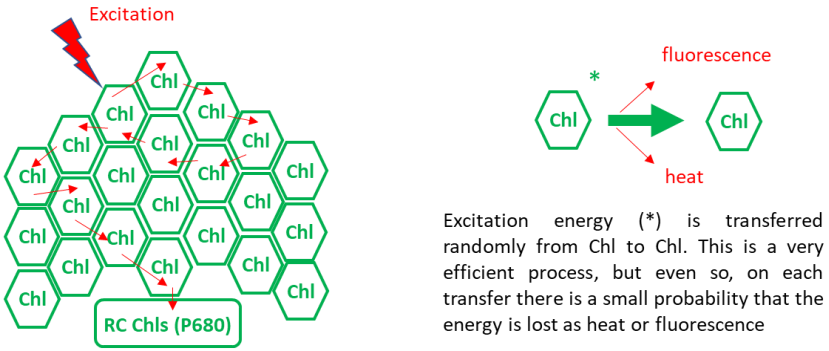

Fig. 2-7: Random walk concept illustrated for the antenna of PS II.

On each energy transfer step there is a small probability that the energy is lost as fluorescence or heat. This means that the fluorescence yield is a function of the lifetime of the excitation energy.

Advanced noted - Historical background

For many years it was essentially impossible to determine the F_0 intensity using high intensity continuous light (which is needed to determine the F_M value). For such measurements a time resolution of 10-20 µs or less is necessary. Scientists created a dark-to-light transition with shutters which had a full opening time of 0.8-2 ms, which was almost a factor 100 too slow for a correct F_0 determination.

There were two solutions for this problem: 1. The use of flash measurements, which allow a combination of low F_0 light with saturating flashes (e.g. laser or xenon flashes); 2. The use of low light intensities.

Xenon or laser flashes are thought to be saturating, i.e., they can reduce Q_A in all PS II reaction centers within microseconds. That

the F_M induced by such single turnover flashes was considerably lower than the F_M induced by a long pulse of light was only discovered in the eighties and ignited in the nineties a polemic about the question what the true F_M was. The topics studied using flashes are quite different, though. The second solution (low light intensity) slowed the kinetics of the fluorescence rise down, allowing a better estimation of F_0, but this had the disadvantage that the maximum measured fluorescence was much lower than F_M. This, for example, led to an overestimation of the fraction of inactive PS II reaction centers. The low, non-saturating, light intensity closed all inactive PS II reaction centers and only a part of the active PS II reaction centers.

The first, commercially available, solution for the F_0 problem was the pulse amplitude modulated (PAM) approach. Instruments using this principle were commercialized by Heinz Walz GmbH. This method separates modulated measuring light (weak enough to measure F_0) and actinic light, which can be made strong enough to induce F_M. At the same time this method made fluorescence measurements insensitive to external light sources.

2.2.5 Advanced note – PS II and PS I fluorescence properties

Variable fluorescence is thought to be largely emitted by PS II and this simplifies the interpretation of Chl *a* fluorescence measurements greatly. Given that PS II and PS I are so similar, how can we explain this difference? There is no consensus on this topic in the literature, and there are scientists that claim that PS I does emit a considerable amount of variable fluorescence. We can get an idea, though, when we look at the behavior of both reaction centers in the light. P680, the reaction center chlorophylls of PS II,

has been shown to be a very efficient quencher of fluorescence in the oxidized state. The state $P680^+Q_A^-$ shows F_0 level fluorescence (or lower; Steffen et al. 2005). However, only under special conditions some accumulation of $P680^+$ occurs that due to its short lifetime very quickly disappears again. $P700^+$, on the other hand, is a species that very easily accumulates. Under high light conditions in the direction of 100% $P700^+$ can accumulate, whereas at the same time the acceptor side of PS II remains largely reduced. If, by analogy with $P680^+$, $P700^+$ is also an efficient quencher of Chl *a* fluorescence, it is logical that there is little PS I variable fluorescence under many conditions (see Byrdin et al. 2000, Steffen et al. 2005).

2.2.6 PAM principle

The term pulse amplitude modulation (PAM) refers to the fact that the instrument measures only the fluorescence amplitude induced by µs measuring pulses of constant height. When PS II reaction centers close (P becomes 0), the fraction of the absorbed light re-emitted as Chl *a* fluorescence (F) increases. That is, the quantum yield of fluorescence increases. It is this change in the fluorescence quantum yield that the measuring pulses detect. Fluorescence induced by continuous light is ignored. The fluorescence detector is blind for the Chl *a* fluorescence induced by (strong) continuous light sources, which can be used to close all PS II reaction centers, without affecting the fluorescence measurement.

In Fig. 2-8 this measurement principle is illustrated. A 'false signal' that does not change the yield of the fluorescence emission induced by the measuring pulses will not be registered: the recorded signal does not change in amplitude. The same is true for stray light reaching the detector. Stray light increases the fluorescence signal but does not change the yield of the fluorescence emission induced by the measurement pulses, therefore, it does not affect the recorded fluorescence signal.

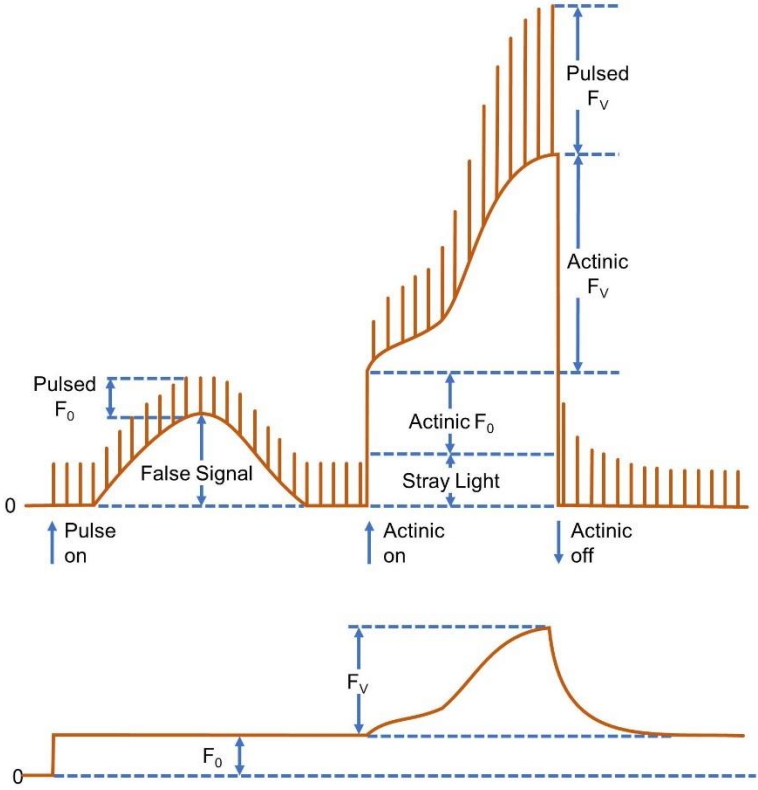

Fig. 2-8: Illustration of the PAM measurement principle.

In the top half of the figure it is demonstrated that only states/situations that increase the fluorescence amplitude (= fluorescence yield) induced by the measuring pulses (the spikes) result in an increase of the PAM fluorescence signal. Only a yield change is recorded. A false signal is e.g. stray light reaching the detector; actinic F_0 or F_V refers to the fluorescence emitted in response to continuous light by open and closed PS II reaction centers respectively, whereas pulsed F_0 or F_V refers to the fluorescence emitted in response to the measuring light pulses by open and closed PS II reaction centers, respectively. Credits: Dr. Ulrich Schreiber

2.2.7 Conventional fluorescence

A few years later, the on/off properties of Light Emitting Diodes (LEDs) started to become good enough for an alternative solution of the F_0 problem: shutterless direct fluorescence measurements. The first instruments of this type had an effective time resolution of 50 µs, which was still a bit too slow for reliable F_0 measurements, but the last 20 years also such instruments can reliably measure F_0 under high light intensities. Modern Walz instruments also use LEDs for all light sources.

2.2.8 Comparison PAM approach with LED-based conventional fluorescence method

In the case of conventional fluorescence measurements, the fluorescence intensity depends linearly on the actinic light intensity. If you do fluorescence measurements at different light intensities, it is necessary to correct for light intensity dependent changes in the fluorescence intensity to make measurements comparable (by dividing by F_0 or the light intensity). Fig. 2-9 illustrates this and shows at the same time that at low actinic light intensities the signal quality of conventional fluorescence measurements decreases, whereas it is insensitive to the actinic light intensity in the case of modulated measurements. As noted above these measurements are sensitive to interference by external light sources.

In the case of conventional fluorescence measurements, the light is off in darkness. This means that there is no low intensity measuring light to monitor F_0 in the absence of actinic light and, therefore, no fluorescence is measured.

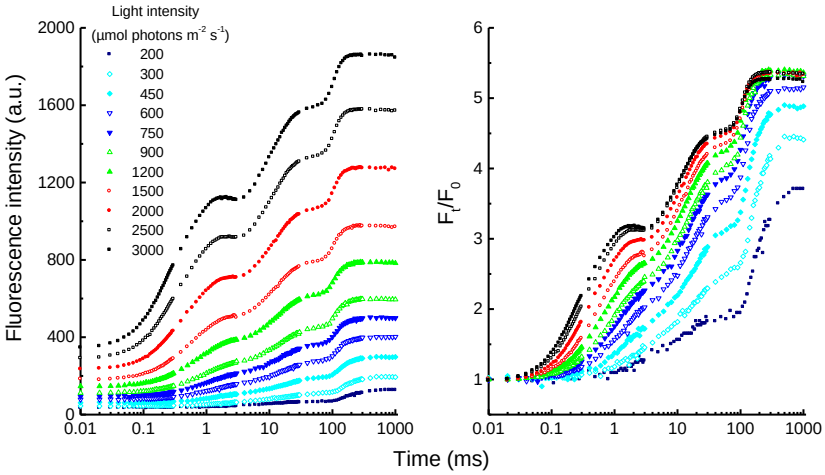

Fig. 2-9: Light intensity dependence of fluorescence induction curves measured on tobacco leaves using a direct fluorescence technique.

The measurements demonstrate that such curves, measured at different light intensities, are difficult to compare and require for that purpose a normalization step. The data also show that signal quality decreases as the light intensity is lowered.

However, the PAM technique also has a few intrinsic weak points. All visible light is actinic, that means that it can induce charge separations and forward electron transfer. If it wouldn't be actinic, we wouldn't be able to measure Chl *a* fluorescence emission. By using low effective light intensities this problem can be reduced so much that the actinic effect becomes insignificant. It will not become 0 though.

Experiment 4.1.3 demonstrates that the actinic effect of the measuring light depends on both light intensity and frequency of the measuring light. When doing PAM measurements, it is important to understand that there is a tradeoff between the measuring light intensity, which determines the noise level, the frequency, which

determines the time resolution and the tolerance of the user towards noisy signals.

Advanced note – Over time several strategies have been developed to cope with actinic measuring light. From the start, a low frequency (low time resolution, little actinic effect) has been used for F_0 measurements and on turning on the actinic light the frequency is increased (higher time resolution, stronger actinic effect, but see next point). Modern PAM instruments add the effective measuring light intensity to the actinic light intensity and only switch to a high measuring frequency above a certain setting of the actinic light. The actinic effect of the measuring light can be especially relevant when doing induction experiments (for example sigma determinations) or measurements of samples inhibited by e.g. the PS II inhibitor DCMU. Measuring light causes a mixing of S-states (= redox states of the oxygen evolving complex) and a PS II acceptor side with 50% Q_B^- (the equilibrium (1:20 at a stroma pH of 7.5) between Q_A and Q_B translates in this case into 2.5% Q_A^- and 47.5% Q_B^-; Diner 1977). To enable the measurement of induction kinetics, trigger and script files are used, which define the experimental conditions and timing of the measurement. That way, the measuring light can be turned on just before turning on the actinic light and starting the measurement. Measuring light also poses a problem when determining fluorescence decay kinetics following a single turnover flash or on turning off the actinic light. Here, the actinic effect can be reduced by using the option to reduce the measurement frequency logarithmically, present in the software of several Walz instruments.

In addition, the maximum frequency of the measuring light puts a maximum on the achievable time resolution. For the MULTI-COLOR-PAM the maximum frequency is 400 kHz translating into

a theoretical maximum time resolution of 2.5 µs. In practice, the time resolution is lower due to the application of signal smoothing and induction effects. This maximum time resolution may become an issue when the experimenter wants to resolve the fluorescence kinetics at very high light intensities.

In summary, the properties of PAM instruments are particular advantageous when doing quenching analyses or making Light Curves. When determining fast fluorescence induction kinetics, non-modulated fluorescence measurements have advantages relative to modulated techniques. The best of both worlds would be an instrument that combines both techniques.

The JUNIOR-PAM has a limited time resolution due to measuring pulse frequencies of 5-10-15-20-25 and 100 Hz (one pulse per 40-200 ms and 10 ms, respectively). This means that the JUNIOR-PAM can only be used for the measurement of relatively slow photosynthetic processes and for experiments like Quenching Analyses and Light Curves.

2.2.9 Abiotic stress

Chl *a* fluorescence is used extensively in abiotic stress studies. Reading the literature, one could get the impression that the parameter F_V/F_M (the maximum PS II quantum yield) can monitor almost any form of stress. However, that is not true. The parameter F_V/F_M is only affected by stress conditions that affect PS II, such as photoinhibition, UV and heat stress. Drought and salt stress, on the other hand, under most conditions, do not affect PS II. However, in many cases other fluorescence parameters can be found in the literature to probe stress conditions that do not affect PS II, e.g. parameters related to the steady state that probe photosynthetic activity.

2.2.10 PAM measurements, the Quenching Analysis and the Xanthophyll cycle

The xanthophylls: violaxanthin, antheraxanthin and zeaxanthin in higher plants and diadinoxanthin and diatoxanthin in diatoms are closely connected to the history of PAM fluorescence measurements. In many papers on the xanthophyll cycle PAM instruments play an important role. But the inverse is also true: when doing PAM measurements, the xanthophyll cycle should be considered.

Quenching Analysis

The introduction of the first PAM fluorometer was linked to the introduction of a PAM-based quenching analysis (Schreiber et al. 1986). At that moment, a few groups had worked already for several years on methodology to distinguish between the effects of the redox state of Q_A on the Chl *a* fluorescence level (photochemical quenching) and the effects of processes that affect the fluorescence quantum yield by changing e.g. the rate constant for heat emission (Non-photochemical quenching) (Krause et al. 1982, Bradbury and Baker 1984, Quick and Horton 1984). The introduction of PAM-instruments allowed a considerable improvement of the methodology (a combination of low intensity modulated measuring light and saturation pulses) and this approach quite quickly became and still is the standard.

As discussed in section 0, with a pulse of strong light it is possible to reduce all Q_A and reduce P [photosynthesis] to 0. The saturation pulses can separate photochemical (qP) and non-photochemical quenching (abbreviated as NPQ when calculated as F_M/F_M'-1 or qN when calculated as 1-F_V'/F_V). Photochemical quenching is related to the redox state of the electron transport chain and non-

photochemical quenching reflects all processes that cause a lowering of F_M.

A lowering of F_M can have several reasons. Light stress at room temperature (even more under chilling conditions) causes photoinhibition of PS II reaction centers. Photoinhibited PS II reaction centers lose their variable fluorescence and this leads to a lowering of F_M.

Another process that is thought to affect F_M is called a state transition. The term 'state transitions' is applied to changes in the PS II antenna size related to the phosphorylation and de-phosphorylation of PS II antenna units called light harvesting complexes II (LHCIIs). This affects the PS II antenna size. Changes in the PS II antenna size also affect F_M. A state 1 to state 2 transition will make the PS II antenna size smaller and thereby cause a lowering of F_M. However, recent research has cast doubt on the strict relationship between phosphorylation of LHCII and antenna transfer between PS II and PS I under natural conditions (e.g. Wientjes et al. 2013).

A third process affecting F_M is formed by chloroplast movements (Fig. 2-10). Relatively 'strong' blue light (>20 µmol photons m^{-2} s^{-1}) induces an avoidance response whereby chloroplasts move to the side walls of the palisade parenchyma cells reducing light absorbance and decreasing the measured F_M. These three processes are considered part of NPQ/qN, although there is no quenching, just less fluorescence.

During light acclimation NPQ/qN is induced, whereas on turning off the light there is a gradual relaxation/recovery during which NPQ/qN gradually goes to 0. This process of NPQ/qN relaxation often shows three kinetic phases that have been called qE (energy quenching), qT and qI, of which only the major component (qE)

has been characterized in detail. The other two components probably reflect mixtures of more than one process. qE affects the properties of the PS II antenna (it reflects an increase of the probability that excitation energy is lost as heat), reduces the fluorescence yield and, as a consequence, lowers F_M.

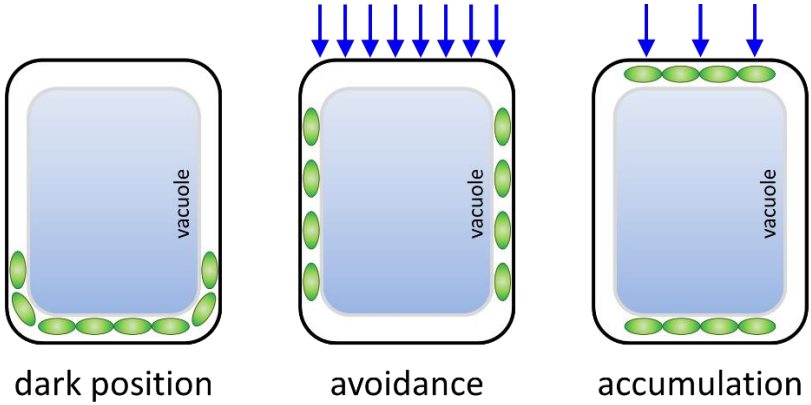

Fig. 2-10: Schematic representation of blue-light-induced chloroplast movements in *Arabidopsis* mesophyll cells.

The position of the chloroplasts within the cell is determined by the blue light intensity (dark adapted, high fluence of blue light, low fluence of blue light; adapted from Wada 2013).

A lot of energy has been invested in the unraveling of the regulatory mechanism underlying qE. It has been established that the driving force behind this mechanism is an acidification of the lumen. The lumen pH depends on the balance between the processes responsible for the release of protons into the lumen (splitting of water by PS II, re-oxidation of PQH_2 by the cyt b6f complex, Q cycle and potentially chlororespiration and cyclic electron transport around PS I) and the consumption of these protons by

ATPsynthase, which in turn depends on ATP consumption by metabolic processes like the Calvin-Benson cycle.

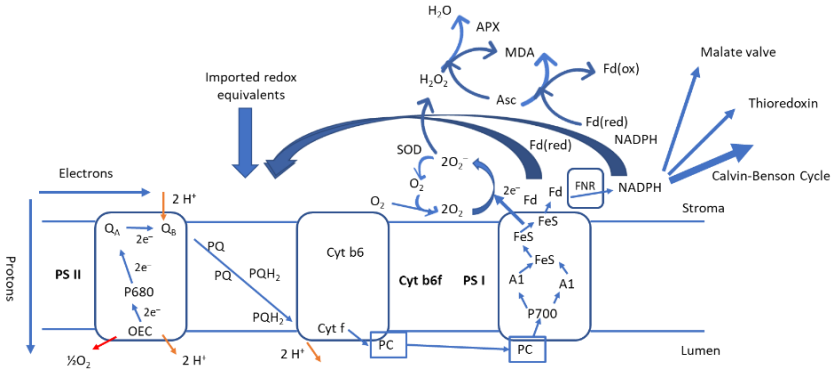

Fig. 2-11: A selection of possible electron transfer reactions (mainly) on the PS I acceptor side that affect the electron to proton ratio.

For a description of the figure see the text below. SOD = superoxide dismutase, APX = ascorbate peroxidase, O_2^- = superoxide, H_2O_2 = hydrogen peroxide, Asc = ascorbate, MDA = malondialdehyde.

In Fig. 2-11 an overview is given of reactions that can increase the proton to electron ratio (and thereby the potential ATP to NADPH ratio). This overview illustrates the impressive network of electron pathways that mainly start on the acceptor side of PS I. Under certain conditions (e.g. chilling, fluctuating light) the PS I acceptor side may become highly reduced (all Fd reduced). In that case, the FeS clusters can donate their electrons to O_2, which results in the formation of superoxide (O_2^-). Oxygen radicals are reactive and can destroy the PS I acceptor side and cause lipid peroxidation. Via superoxide dismutase (SOD) and ascorbate peroxidase two superoxide molecules are first turned into O_2 and H_2O_2 by SOD and subsequently in water by ascorbate peroxidase (APX). This is called the water-water cycle (Asada 1999) and its purpose

is 1. to detoxify the produced oxygen radicals and 2. to waste electrons. Further, two pathways have been described for cyclic electron transport. One pathway uses reduced Fd for the reduction of PQ molecules and the other pathway uses NADPH for the same purpose. Reduction of the PQ pool can also be caused by electrons from the mitochondria (this is called chlororespiration; e.g. Bennoun 1982, Yoshida et al. 2008) and at the same time electrons can be exported to the mitochondria and peroxisomes via the so-called malate valve (e.g. Fridlyand et al. 1998). Thioredoxins are regulatory molecules that determine the activation state of several Calvin-Benson cycle enzymes, ATPsynthase and the malate valve (Scheibe 1990, Knuesting and Scheibe 2018). The relative importance of all these processes is hotly discussed in the literature. However, many experiments have shown that under standard conditions, in the steady state, the Calvin-Benson cycle is the dominant electron sink.

A low lumen pH has several consequences: it sensitizes the PS II antennae to qE and it activates an enzyme called violaxanthin de-epoxidase (VDE) responsible for the de-epoxidation of violaxanthin to zeaxanthin via the intermediate antheraxanthin. It was subsequently discovered that in the PS II antenna there is a protein called PsbS that mediates the effect of a low lumen pH to the PS II antenna. Diatoms have a two-component system in which the xanthophyll cycle only consists of diadinoxanthin and diatoxanthin.

Advanced note – Here, it is important to realize that there is a difference between open and closed reaction centers. In open reaction centers the lifetime of the excitation energy is short and this means that the qE quenching mechanism has not much time to quench the excitation energy. In closed reaction centers the lifetime of the excitation energy is considerably longer and there is,

therefore, considerably more time for zeaxanthin/low lumen pH/protonated psbS to quench the excitation energy and emit it as heat (see Fig. 2-12).

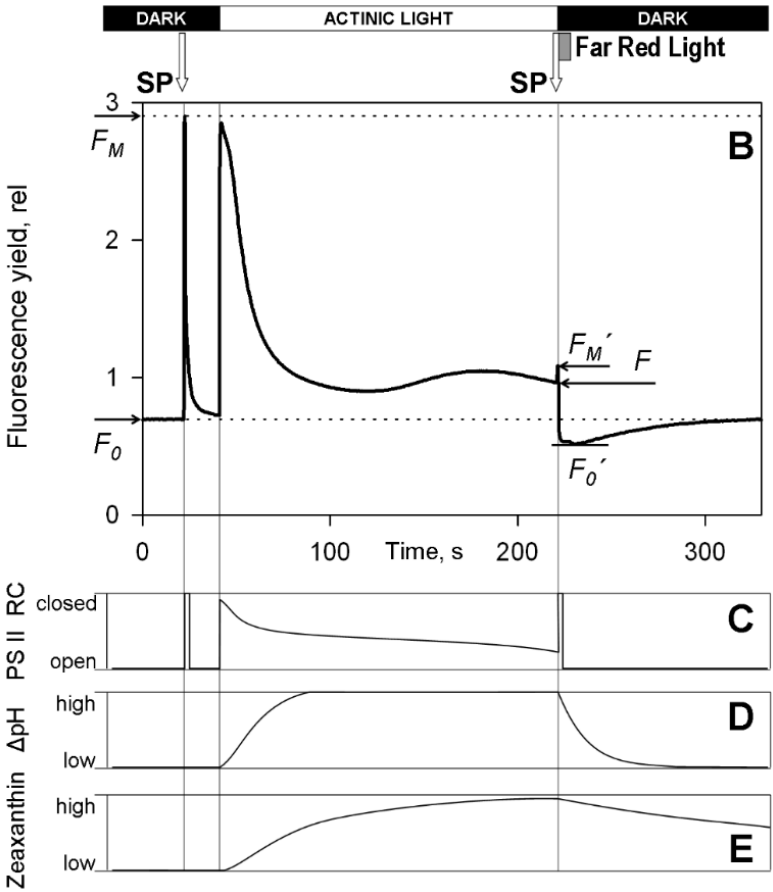

Fig. 2-12: Relationship between the induction of photosynthesis, the quenching analysis and its parameters: the closedness of PS II reaction centers (qP) and the induction of the ΔpH and of zeaxanthin.

NPQ, more specifically qE, gives us information on closed reaction centers (quenching of F_M). To be able to say something about the effect of qE on open reaction centers, we should determine the extent of F_0 quenching. F_0 quenching reflects the ability of zeaxanthin/low lumen pH to quench excitation energy in open PS II reaction centers. The beauty of the qE quenching mechanism is that it allows an effective quenching of excitation energy in closed reaction centers while keeping the quantum yield of open reaction centers high.

2.2.11 References

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3 Working with the JUNIOR-PAM

3.1 Components and assembly of the JUNIOR-PAM

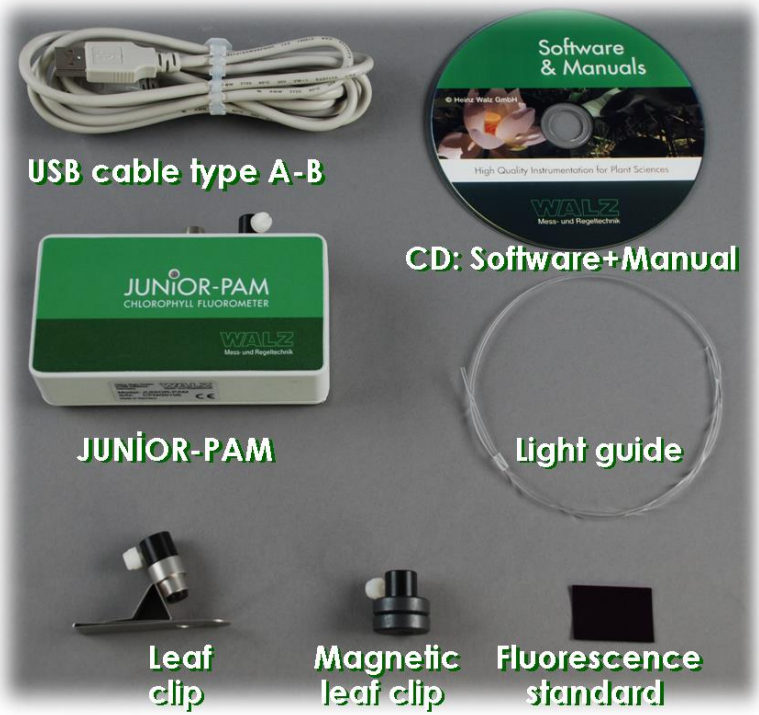

Fig. 3-1: JUNIOR-PAM components.

3.1.1 Assembly of the JUNIOR-PAM fluorometer

Unpack fiber optics. Carefully insert the fiber end with the silicone sleeve into the fiber port of the JUNIOR-PAM (Fig. 3-3) until silicone sleeve and fiber port get in contact. Frequently, the fiber encounters a resistance during insertion: if this is the case, remove the fiber, carefully straighten the fiber by hand, and insert again. Do not forcible overcome the resistance. Finger-tighten the plastic screw of the light guide port.

Insert free end of light fiber into top of the fiber 'port' of the magnetic leaf clip (Fig. 3-4) until the fiber tip is in contact with the sample-facing surface of the fiber port. Finger-tighten the plastic screw of the magnetic leaf clip. Now, the distance between fiber tip and the surface of a sample held by the magnetic leaf clip is 1 mm.

Plug the type B plug of the USB cable in the JUNIOR-PAM, and the type A plug in a computer running with Microsoft Windows XP, Vista or 7/8/10 operating systems (Fig. 3-2). At this point; the top side signal LED of the JUNIOR-PAM should flash green at a frequency of 1 Hz, and, at the end of the fiber, blue measuring light should be visible.

To computer

To fluorometer

Fig. 3-2: USB cable.

Fig. 3-3: JUNIOR-PAM light guide port and sockets.

Fig. 3-4: Magnetic leaf clip.

3.1.2 Installation of WinControl-3 software

Depending on the type of CD-ROM delivered with the JUNIOR-PAM you have to start installation with article a) or article b).

a) Your <Software & Manuals CD-ROM> contains only a setup file (e. g., <WinControl-3-3.13-Setup.exe>) and the JUNIOR-PAM manual in PDF file format.

Double click on the setup file and follow instructions. The setup routine will create the folder <WinControl-3> containing WinControl-3 software in the <c:\Program Files> directory. Further, a USB serial converter driver will be installed, and shortcuts to the WinControl-3 software will be created in the <Program> section of the Windows <Start> menu and elsewhere, depending on your selection.

b) Your <Software & Manuals CD-ROM> contains the complete collection of the Walz Software & Manuals. In this case, the CD starts the default internet browser of your computer. (If automatic browser start fails, double-click on <index.html> in the root directory of the <Software & Manuals> CD-ROM.)

Choose <Fluorescence Products> ➔ <JUNIOR-PAM> ➔ <PC software WinControl-3>. Clicking on <WinControl-3> will start software installation as described above.

With the fluorescence standard or a green leaf in the magnetic leaf clip, and “Meas. Light” and “Rec. Online” checked, the chart typically displays values greater than 200 digital units. Click <Autoscale> if data are not visible. In case the actually measured values are clearly lower than 200 digital units, make sure that the silicone sleeve of the optical fiber is in contact with the fiber port (is inserted correctly).

3.1.3 Operation of the JUNIOR-PAM

To understand all the options of the JUNIOR-PAM we want to suggest the user of this booklet to read Chapter 5 of the JUNIOR-PAM manual. Here, we limit ourselves to the points relevant for the experiments described below.

There are separate graph windows for recordings of Induction Curves, Light Curves and Saturation Pulse kinetics. However, the manipulation of these graph windows is in each case the same.

3.1.4 What are the most important settings and parameters for the experimental chapter?

When working with the JUNIOR-PAM what are the instrument variables that can be varied? Important parameters are the intensity

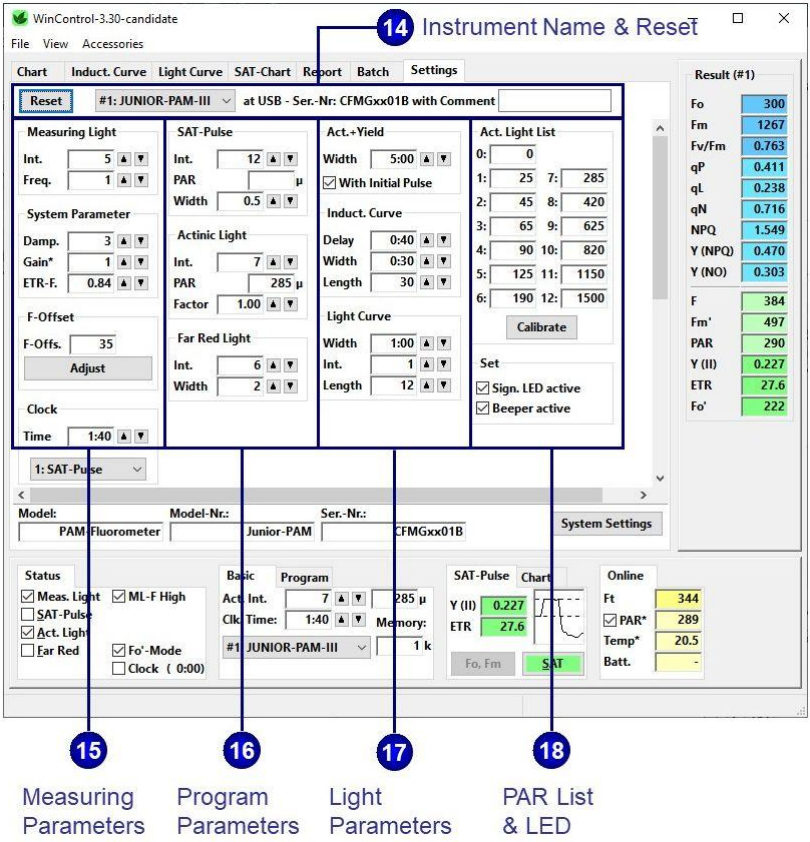

Fig. 3-5: The Settings window of the JUNIOR-PAM.

Panels 15 and 16 are relevant for the experiments described here. In panel 15 the measuring light intensity and frequency can be adjusted. For changes in the saturation pulse and far-red light intensity and width as well as the actinic light intensity the experimenter should go to panel 16. In panel 17, induction and light curves can be defined: for Induction Curves the parameter <Delay> defines the time between the <F0,FM> and the start of the Induction Curve, whereas the parameter <Width> defines the time between saturation pulses, and <Length> the total number of saturation pulses. Light Curves are defined by <Width>, which is the length of each light step, <Int.> defines the light intensity

of the ac- with which the Light Curve starts, and <Length> defines the number light intensities of which the Light Curve exists.

tinic (white or blue) light with which photosynthesis is driven, the far-red light with which a reduced electron transport chain can be quickly oxidized again (see experiment 4.1.5), but also the length of periods of illumination and the periods of darkness between illuminations (Fig. 3-5).

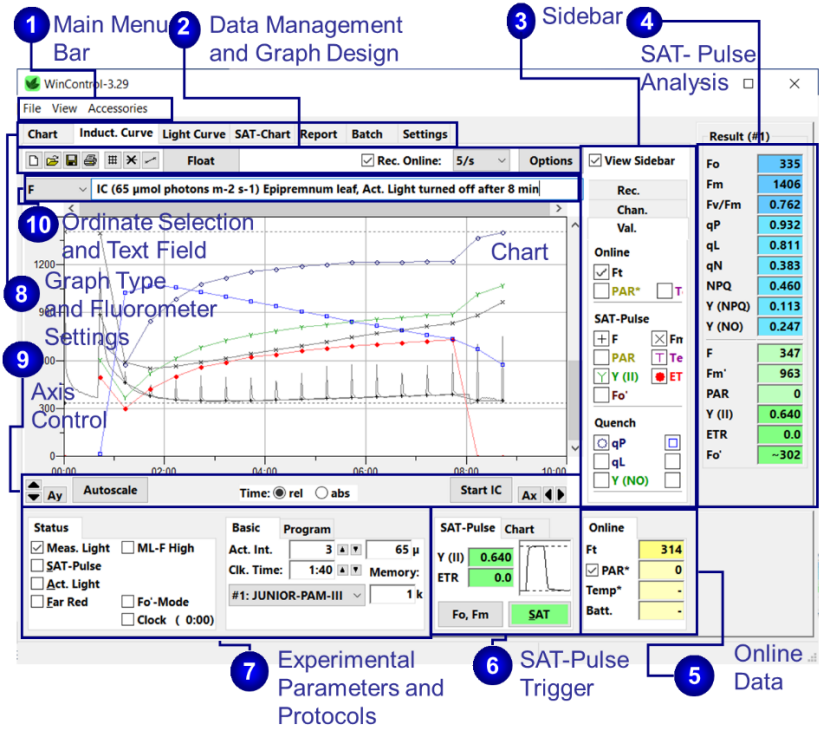

Fig. 3-6: Induction Curve window (opening window when running the software).

To keep the data on scale, the <Autoscale> button in panel 9 is the easiest option. The value-tab of panel 3 allows the experimenter to choose

the parameters to be shown in the graph window, where F represents the measured fluorescence signal. With the <F0,FM> button in panel 6 the F_0 and F_M values of a dark-adapted leaf can be determined. In panel 9 also the <start IC> and <start LC> buttons can be found, that initiate the Induction Curve and Light Curve protocols.

At any point during such a sequence of periods of light and darkness the state of the photosynthetic electron transport chain can be probed with a saturation pulse of light, the most important tool available to people making fluorescence measurements with the JUNIOR-PAM.

The output of such measurements is the time dependence of the Chl *a* fluorescence intensity, but what this means depends strongly on the question what went before (the memory/acclimatization effect is worked out in experiment 4.1.10). Induction measurements provide us with other information than steady state measurements. Important fluorescence parameters are: F_0, F_0', F_M, F_M', F, F_V and the fluorescence ratios F_V/F_M, F_V'/F_M', qP, NPQ (Fig. 3-6).

The outcome of fluorescence measurements depends on the species studied (e.g. C3 or C4 (like maize or sorghum) plants, gymnosperms (e.g. needle trees), mosses, green algae, cyanobacteria, diatoms, etc.) and on the conditions under which these species are studied (e.g. abiotic stress conditions like drought, heat stress, salt stress, photoinhibition, cold stress, etc. or photosynthetic mutants).

With these variables the number of experiments that can be designed and carried out is limitless. In Chapter 4 illustrations of different aspects of fluorescence measurements and their application are given.

4 Experimental section

With the set of experiments worked out in this chapter a range of phenomena is illustrated that can be probed and characterized by Chl *a* fluorescence. These experiments will also show the experimenter that a detailed interpretation of the data is only possible thanks to the foundations laid by biochemical research.

The experiments worked out in the booklet have been divided in several themes: 1. experiments that give insight in the properties of Chl *a* fluorescence and the measurement of this signal, 2. experiments giving insight in the properties of the photosynthetic apparatus, 3. experiments related to acclimation (sun/shade) and the optical properties of leaves, 4. experiments related to abiotic stress.

Below, a list of the experiments described in this chapter is given. Each experiment has been classified. The term ‘technical’ refers to topics that are relevant to the measurement of Chl *a* fluorescence. The term ‘basic’ refers to topics that are relevant for understanding of fluorescence measurements. The term ‘advanced’ does not so much refer to difficult, but to topics that are nice to know, but are not directly relevant to simple fluorescence measurements. The term ‘physiology’ refers to topics that teach us something about the biology of the studied organism. Finally, the term ‘settings’ refers to topics that help to decide which settings should be chosen when doing particular measurements.

In the appendix of this booklet example measurements for several of the experiments treated here are given.

Table 2: Overview on Experiments.

	Experiment	Basic	Technical	Settings	Advanced	Physiology
4.1	Properties of Chl *a* fluorescence (measurements)					
4.1.1	Testing fluorometer function using the Walz fluorescence standard	☒	☒	☐	☐	☐
4.1.2	Signal scaling	☒	☒	☒	☐	☐
4.1.3	Measuring light (ML) intensity	☒	☒	☒	☐	☐
4.1.4	SP pulse length	☒	☒	☒	☐	☒
4.1.5	Far-red light	☐	☒	☐	☒	☒
4.1.6	F0'-calculation	☐	☒	☒	☒	☐
4.1.7	The FV/FM-value	☐	☐	☐	☒	☒
4.1.8	High light versus low light	☐	☒	☒	☒	☐
4.1.9	Attached and detached leaves	☐	☒	☐	☒	☐
4.1.10	Memory/Long-term effects	☐	☐	☐	☒	☒
4.1.11	Chloroplast movements	☐	☒	☐	☒	☒
4.1.12	State transitions	☐	☐	☐	☒	☒
4.2	Properties of the photosynthetic apparatus					
4.2.1	The acceptor side of PS I	☐	☒	☐	☒	☒
4.2.2	Quenching below F0	☐	☒	☐	☒	☒
4.3	Sun/shade acclimation and leaf optical properties					
4.3.1	The sidedness of leaves	☐	☒	☐	☒	☒
4.3.2	Shade versus sun leaves	☐	☐	☐	☒	☒
4.4	Abiotic stress					
4.4.1	Photoinhibition	☐	☐	☐	☒	☒
4.4.2	Heat stress	☐	☐	☐	☒	☒
4.5	Field experiments					
4.5.1	Canopy variability around a big tree	☐	☐	☐	☒	☒
4.5.2	Canopy gradients in a maize or wheat field	☐	☐	☐	☒	☒
4.5.3	Drought stress	☐	☐	☐	☒	☒

4.1 Properties of Chl *a* fluorescence (measurements)

4.1.1 Testing fluorometer function using the Walz fluorescence standard

What is the purpose of the fluorescence standard (foil, dark reddish color) that is provided with our instruments?

When we put a leaf in a leaf holder and then turn first the measuring light on and then the actinic light, changes in the fluorescence intensity are observed but these changes are an interplay between the light sources and the status of that leaf. It is not possible to determine if the instrument works correctly. With the fluorescence standard it is possible to determine the response time of the light sources, the stability of the light intensity produced by the LEDs and the distance between sample and fiber (or fiber and control unit).

With the following experiment the difference between foil and leaf measurements, and what it teaches us about pulse amplitude modulated fluorescence measurements, is illustrated.

Experimental time: ~30 min

Material: a C3 leaf and the fluorescence standard

The magnetic leaf holder of the JUNIOR-PAM consists out of two parts of a magnet between which the sample is placed. The top part of the leaf holder has a hole for the fiber (Fig. 3-4).

The applied light intensity and measured fluorescence intensity depend on the distance between the fiber and the leaf and/or the distance between the fiber and the instrument. The fiber can be inserted up to the sleeve into the instrument. The sleeve is there to prevent damage.

Experiment: Place the fluorescence standard in the leaf holder. The software records continuously the measured fluorescence value in the chart window. Turn the measuring light on (ML 1, gain 1) by checking the Meas. Light in the status window. Increase every 10-20 s the ML intensity (1, 2, 4, 6, 8, 10, 12). Each time the ML intensity is increased, the measured fluorescence intensity increases as well. And this increase is not rectangular but has certain kinetics. Then turn on the actinic light (by checking the act. light box) and then a saturation pulse (by checking the Sat-Pulse box) to establish that these actions do not affect the measured fluorescence intensity and to illustrate the PAM technique (Fig. 4-1). To demonstrate the effect of the distance between fiber and instrument and/or fiber and sample pull the fiber a little bit out of the instrument and see what this does to the Ft value on the screen in the online box.

Then, repeat the experiment for the leaf of the C3 plant.

Compare the 2 sets of measurements and describe the differences.

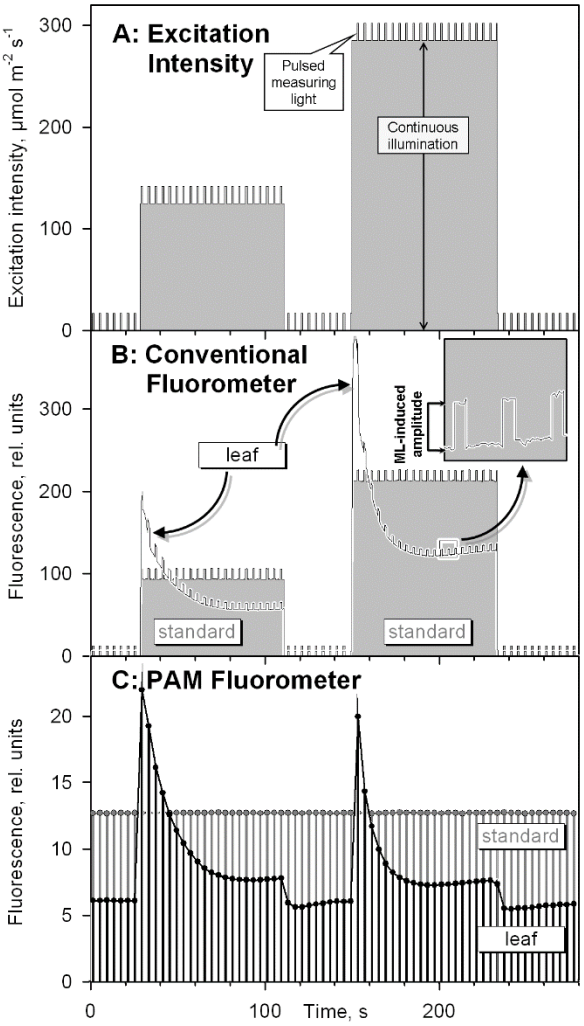

Fig. 4-1: Comparison of the fluorescence properties of the fluorescence standard (foil) and a leaf.

4.1.2 Signal scaling

Key parameters in Chl *a* fluorescence measurements are the F_0 and F_M values. To get meaningful measurements both these parameters should be kept on scale. This means that correct values for measuring light and gain have to be chosen. We know that the maximum ratio between F_0 and F_M is ~6 (F_V/F_M ~0.83, see 4.1.7) if the fluorescence intensity is measured at wavelengths longer than 700 nm. This means that the F_0 value should not exceed 1/6 of the maximum scale, unless the experimenter knows that the F_V/F_M value is much lower than this maximum value. The important point here is that the experimenter can use the F_0 value to judge if the measurement will stay on scale.

Experimental time: ~1 h

Material: a leaf of any plant that interests the experimenter that has been kept in darkness for at least an hour.

Experiment: determine the measuring light intensity and gain dependence of the F_0 and F_M values and see where the signal goes off-scale. The experiment will consist of a set of saturation pulses. First it is important to establish how much time is needed between pulses (in the absence of actinic light) to get each time the same $(F_M-F_0)/F_M$ value (use here 5 min). Then start the actual experiment. Begin with gain = 1 and click each time on the <Fo,Fm> button after setting ML to, respectively, 1, 2, 4, 6, 8, 10 and 12 (using the each time the time interval established before). Note each time the F_0 and F_M and F_V/F_M values. Repeat the same procedure for gain = 2 and gain = 4 and make graphs of the F_M intensity against ML for the three datasets, to get a feeling for the relationship between the signal intensity and the different settings.

The obtained relationships are not valid for plant species in general. As shown by Dinç et al. (2012) the amplitude of the variable fluorescence is quite sensitive to the PS II antenna size.

It is important to be aware of the fact that the fluorescence signal can go off-scale, but it is also important to know at which value the maximum is reached (for the JUNIOR-PAM the signal will go off-scale above 4000 units) and to recognize measurements where the signal went off-scale. This will truncate the top of the fluorescence transient.

References:

Dinç E, Ceppi MG, Tóth SZ, Bottka S, Schansker G (2012) The Chl a fluorescence intensity is remarkably insensitive to changes in the chlorophyll content of the leaf as long as the chl a/b ratio remains unaffected. Biochim Biophys Acta 1817: 770-779

4.1.3 Measuring light (ML) intensity

To measure fluorescence, a probe pulse has to induce some charge separations. In the case of measuring light, the integrated light intensity of the measuring pulses is so low that the excitation rate stays below the rate of forward electron transfer from Q_A to Q_B and there is a small stable actinic effect. The key-criterion for a good measuring light intensity is that it induces a stable fluorescence intensity. It has to be kept in mind that the integrated light intensity of (modulated) ML is determined by two factors: the frequency and the intensity. The frequency determines the time resolution of a measurement. If nothing happens the frequency can be low, but the moment fast kinetic changes occur the frequency has to be high enough to resolve these kinetic changes. A frequency of 1 Hz means one measuring pulse per second and, therefore, a maximum time resolution of 1 s; at 1000 Hz, there is 1 pulse per ms and we have a maximum time resolution of 1 ms; at 100 kHz we have 1 pulse per 10 µs and a maximum time resolution of 10 µs. It is of course logical that the integrated light intensity at 100 kHz is 100,000 times higher than the integrated light intensity at 1 Hz. The right ML intensity will always be a compromise between the level of noise, the required time resolution and the amplitude of the induced effect. A small fluorescence change will more easily drown in the noise than a large fluorescence change. The goal of this experiment is that the experimenter gets a feeling for the interaction between Chl *a* fluorescence and measuring light intensity.

Experimental time (including the construction of the figures): 1-2 h

Material: a leaf of any plant the experimenter is interested in.

Experiment: Chose the lowest measuring light intensity in <Settings> and 5/s = 5 Hz in the <Chart> Window, turn the ML on, measure for 1 min and note down the Ft-value found in the bottom right window of the screen, increase the measuring light intensity, continue measuring. Repeat the procedure and after intensity 4, continue with 6, 8, 10, etc. For the JUNIOR-PAM, decrease the frequency to 1 Hz (if the measurements are made with another Walz fluorometer, increase the frequency to 1000 Hz) and repeat the experiment. Make a graph of the fluorescence intensity determined after each min as a function of the measuring light intensity for both 100 and 1000 Hz.

Questions: which measuring light intensities for both frequencies gives still a stable F_0 value and at what measuring light intensity is the noise level still acceptable to the experimenter?

4.1.4 SP pulse length

The saturation pulse is a key element of a quenching analysis (and several other fluorescence analysis approaches as well). The correct length of an SP pulse follows (theoretically) from the definition of an SP pulse. An SP pulse should be neutral and not affect the subsequent SP pulse. This means in practice that an SP pulse should be long enough to reach F_M or F_M', but it should not induce non-photochemical quenching, it should only probe it.

Experimental time: 1-2 h

Material: an angiosperm leaf for *Experiment 1* and e.g. a *Ginkgo* leaf or pine needles for *Experiment 2*.

Experiment 1: Start the software and go to the <Chart> window. Set the SP pulse length to 200 ms. Turn on the measuring light and after about 10 s give an SP. Change the SP pulse length to 300 ms and give 5 min after the first pulse a second SP. Change the SP pulse length to 400 ms and give 5 min after the second pulse a third SP, etc., increasing the pulse gradually via 500 ms, 600 ms, 800 ms to 1 s. Repeat the experiment starting with a 1 s pulse length.

Questions: was the 200 ms pulse long enough to reach F_M? At which pulse length the F_M of the subsequent pulse starts to become lower? At which pulse length the fluorescence intensity starts to decline again after reaching the maximum value?

A perfect SP is long enough to reach F_M' but not so long that the fluorescence intensity starts to decline again.

Note – As the figure in the appendix shows it was difficult to get completely reproducible results and there was an order effect. It is

possible that the blue light pulses (via blue light receptors) have some secondary effects on the exact F_M level (see 4.1.11).

Advanced note – When the experimenter would read Quenching Analysis literature from the nineties, he/she would notice that 700-800 ms SP pulses where used in most cases. At the time halogen lamps instead of LEDs were used for the SPs. These halogen lamps needed about 300 ms to warm up and reach their maximum light intensity. So, the length of the SP at maximum light intensity was only 400-500 ms.

4.1.5 Far-red light

Photosynthetically active radiance (PAR) is defined as light between 400 and 700 nm. PAR excites both photosystems. Excitation of PS II already starts to decline beyond 680 nm and beyond 700 nm, PS II excitation becomes very limited, however, it does not decline to 0. PS I, on the other hand, absorbs far-red light more efficiently than PS II. In photosynthesis research we make use of this property. Using far-red light, it is possible to pump electrons out of the photosynthetic electron transport chain. Far-red light is used in measurements to reduce the time needed to reach F_0(') following a saturation pulse. In addition, FR prevents a secondary F_0 rise due to cyclic electron transport around PS I and other forms of non-photochemical reduction of the PQ pool.

In the following experimental sequence these effects are explored.

Experimental time: 1-2 h

Material: for the first experiment any C3 plant available and for the second experiment a C4 plant (e.g. *Zea mays*), a C3 plant with high PQH_2 re-oxidation activity (e.g. *Hordeum vulgare* (in general a grass or a sedge) or *Camellia japonica*) and a C3 plant with a relatively low PQH_2 re-oxidation activity (e.g. *Pisum sativum*).

Experiment 1: Start the software and go to the <Chart> window. Actinic light and Far red light can be turned on and off by checking and unchecking <Act. Light> and <Far Red>. a. turn on the measuring light (use the 200 ms time resolution (5/s)), give a saturation pulse <Fo, Fm> and determine the time needed to return to F_0, then b. 3 min later turn the far-red light on (setting 12) and immediately afterwards give a saturation pulse. Again, determine the time needed to reach F_0 (then turn off the FR light). Maybe the experiment can be repeated with several FR-intensities. Then, c.

turn on the actinic light (maybe 400/500 µmol photons m^{-2} s^{-1}) during 5 min. Turn off the actinic light and determine how quickly and how deep the fluorescence falls and see if it increases again after reaching a minimum. Then, d. turn the actinic light intensity on again for about 50 s, turn on the FR light and nearly at the same time turn off the actinic light. Determine the time and the value to which the fluorescence drops. Then, e. turn the actinic light on again for another 50 s, turn the actinic light off, give a saturation pulse (click the different buttons as quickly as possible) and again determine the time to reach F_0'. Finally, f. turn the actinic light on again for 50 s, turn the actinic light off, far red light on and give a saturation pulse.

Determine for which of the foregoing treatments the FR light decreased the time to reach F_0.

Experiment 2: On turning off the actinic light in the steady state the fluorescence intensity drops in the direction of F_0' and then starts to increase again in many cases. These secondary fluorescence rise kinetics in darkness are determined by two factors: on the one hand, the rate of non-photochemical reduction of the PQ-pool by processes like cyclic electron transport around PS I and chlororespiration and on the other hand the PQH_2-oxidase activity. In grasses the re-oxidation rate of the PQ-pool in darkness is rather high with halftimes below 10 s and in some plants below 1 s. In many other plants it is much slower. C4 plants like maize have higher rates of cyclic electron transport than C3 plants.

The experimental protocol is simple: turn the actinic light on (200-400 µmol photons m^{-2} s^{-1}) for 5-10 min, then turn the actinic light off but keep measuring on for another 100 s.

Make a comparison between a maize plant (or any other C4 plant that is available), a barley or *Camellia* plant (C3 plants with high

PQH_2 oxidase activity) and a pea plant (C3 plant with relatively low PQH_2 oxidase activity) with respect to the kinetics of the 'F_0'' value.

Note: when using red excitation light, F_0' calculations (Oxborough and Baker 1997) give quite reliable data (Pfündel et al. 2018).

References:

Gotoh E, Matsumoto M, Ogawa K, Kobayashi Y, Tsuyama M (2010) A qualitative analysis of the regulation of cyclic electron flow around photosystem I from the post-illumination chlorophyll fluorescence transient in Arabidopsis: a new platform for the in vivo investigation of the chloroplast redox state. Photosynth Res 103: 111-123

Munné-Bosch S, Shikanai T, Asada K (2005) Enhanced ferredoxin-dependent cyclic electron flow around photosystem I and α-tocopherol quinone accumulation in water-stressed ndhB-inactivated tobacco mutants. Planta 222: 502-511

Oxborough K, Baker NR (1997) Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and F_V'/F_M' without measuring F_0'. Photosynth Res 54: 135-142

Pfündel EE, Latouche G, Meister A, Cerovic ZG (2018) Linking chloroplast relocation to different responses of photosynthesis to blue and red radiation in low and high light-acclimated leaves of Arabidopsis thaliana (L.). Photosynth Res 137: 105-128

4.1.6 F_0'-calculation

Oxborough and Baker (1997) proposed a formula for the calculation of F_0'. The rationale for this approach was that at the time it was not possible to image this parameter, whereas it was needed for the calculation of the parameters qP and F_V'/F_M'. In a 2018 paper Pfündel et al. evaluated this approach for blue and red excitation light applied to low and high light acclimated plants. The authors found a good match between measured and calculated F_0' values in the case of red actinic light if the data were corrected for the contribution of PS I fluorescence to F_0. For *Arabidopsis* leaves a 24% contribution of PS I fluorescence to F_0 was found that was not sensitive to non-photochemical quenching.

The goal of this experiment is to make a comparison between calculated and measured values of F_0' for a plant of choice. In the JUNIOR-PAM software the so-called F_0-mode can be activated, in which case a saturation pulse is followed by a 3 s pulse of far-red light.

In C3 plants the PS I contribution to F_0 is about 25-30%, whereas it is at least 50% in C4 plants.

Experimental time: ~2 h

Material: a C3 plant and a C4 plant

Experiment (can be combined with 4.1.7): make Light Curves for both types of plants with and without F_0-mode and compare the F_0 and F_0' values as a function of the actinic light intensity and in parallel compare the parameters qP and F_V'/F_M' whose calculation is affected by F_0'.

Question: are the values comparable (for both types of plants)? And if not, how big are the differences?

References:

Genty B, Wonders J, Baker NR (1990) Non-photochemical quenching of F_0 in leaves is emission wavelength dependent: consequences for quenching analysis and its interpretation. Photosynth Res 26: 133-139

Oxborough K, Baker NR (1997) Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and F_V'/F_M' without measuring F_0'. Photosynth Res 54: 135-142

Pfündel E (1998) Estimating the contribution of photosystem I to total leaf chlorophyll fluorescence. Photosynth Res 56: 185-195

Pfündel EE, Klughammer C, Meister A, Cerovic ZG (2013) Deriving fluorometer-specific values of relative PSI fluorescence intensity from quenching of F_0 fluorescence in leaves of *Arabidopsis thaliana* and *Zea mays*. Photosynth Res 114: 189-206

Pfündel EE, Latouche G, Meister A, Cerovic ZG (2018) Linking chloroplast relocation to different responses of photosynthesis to blue and red radiation in low and high light-acclimated leaves of *Arabidopsis thaliana* (L.). Photosynth Res 137: 105-128

4.1.7 The F_V/F_M-value

Kitajima and Butler (1975) derived for a model in which the redox state of Q_A determined the fluorescence rise from F_0 to F_M that the parameter F_V/F_M equates the maximum quantum yield of PSII when all reaction centers are open. This calculation assumes that both F_0 and the variable fluorescence F_V are emitted by PS II only. It was shown that this is not the case and that in C3 plants on average 30% of F_0 is due to PSI fluorescence and in C4 plants this is 50-55%.

The parameters F_V/F_M and F_V'/F_M' are probably the most used fluorescence parameters. They are in many cases used as maximum and effective quantum yield of PS II. In addition, the F_V/F_M is used as a general stress parameter.

Experimental time: from 30 min to several hours

Materials: a C3 and C4 plant (and, if possible, cells from several classes of phytoplankton)

Experiment 1: Place a C3 or C4 leaf in the leaf holder. Go to the <Chart> window. Turn on the measuring light and after about 10 s click on <F_0,F_M> to determine the F_V/F_M value. Note the plant species, plant type and F_V/F_M value down. Repeat the procedure for several C3 and C4 plants.

Question: Are the F_V/F_M values of C3 plants systematically higher than those of C4 plants as expected?

Supplementary experiment 2: Test the effect of thylakoid membrane stacking on the F_V/F_M value.

The F_V/F_M value is also sensitive to features like membrane stacking. In stacked membranes PS II and PS I are kept apart and this prevents interaction. In many aquatic organisms the thylakoid

membranes are less well stacked, there is more interaction between PS II and PS I antennae possible and due to transfer of excitation energy between antennae slow PS II reaction centers loose excitation energy to fast PS I reaction centers causing lower F_V/F_M values. Cyanobacteria that lack stacking and have low PS II to PS I ratios are particularly affected.

If possible, test this by determining the F_V/F_M value for different classes of phytoplankton

Advanced note – As noted above the F_V/F_M value is sensitive to several factors (stacking, PS I fluorescence), however, there are limits to the possible range of values. In general, PS II is functional, or it is inactive, in nature there is not too much in between. If the experimenter would inactivate PS II, illuminating a leaf with high light intensities (photoinhibition), he/she will observe a gradual decrease of the F_V/F_M value. This does not mean that the quantum yield of individual PS II reaction centers gradually decreases. In the case of a stress condition like photoinhibition it is better to look at the measurements in terms of populations of PS II. There are light-inactivated centers that have (largely) lost their ability to emit variable fluorescence (F_V/F_M = ~0) and still completely active centers (F_V/F_M = normal). The measurement averages the contribution of these two populations of PS II, and this leads to a range of F_V/F_M values, reflecting the level of inhibition.

References:

Genty B, Wonders J, Baker NR (1990) Non-photochemical quenching of F_0 in leaves is emission wavelength dependent: consequences for quenching analysis and its interpretation. Photosynth Res 26: 133-139

Kitajima M, Butler WL (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim Biophys Acta 376: 105-115

Pfündel E (1998) Estimating the contribution of photosystem I to total leaf chlorophyll fluorescence. Photosynth Res 56: 185-195

Trissl H-W, Wilhelm C (1993) Why do thylakoid membranes from higher plants form grana stacks? Trends Biochem Sci 18: 415-419

Wientjes E, van Amerongen H, Croce R (2013) Quantum yield of charge separation in photosystem II: functional effect of changes in the antenna size upon light acclimation. J Phys Chem B 117: 11200-11208

4.1.8 High light versus low light

Some scientists have argued that fluorescence measurements should be made at low light intensities, because high light intensities are not physiological. Here, the experimenter should separate two cases. With physiological relevant light intensities, the experimenter can create physiologically relevant states of the photosynthetic apparatus which can then be analyzed. However, if the experimenter wants to analyze a state it is often a good idea to use high light intensities. Take for example the F_M. At physiologically relevant light intensities it is not possible to determine the F_M. We use, therefore, a pulse of strong light. High light intensities make rate limiting steps visible and give better defined fluorescence transients that contain more information that can be analyzed. However, as shown by Schreiber et al. (2019) it should be considered that extremely high light intensities induce additional processes like fluorescence quenching carotenoid radicals that may complicate the analysis.

Experimental time: ~2 h.

Materials: a C3 leaf

Experiment 1: take a leaf, place it in the leaf holder, turn the measuring light on, give every 200 s a 1 s pulse of light with increasing light intensities up to 6000 µmol photons m^{-2} s^{-1}. Repeat the experiment but now with 100 µmol photons m^{-2} s^{-1} actinic light and 10 min of actinic illumination before the start of the measurement. Plot the light intensity dependence of the measured F_M and compare the two cases.

Experiment 2: However, when studying the steady state and the light intensity dependence of the parameters associated with that steady state the situation is different. Make LCs, step length 120

s and analyze in which light intensity range the major changes in the determined parameters occur.

Supplementary experiment 3: If the experimenter has an instrument with which O-I1-I2-P transients can be measured he/she can use a script for the measurement of a 300 ms induction curve at high time resolution. This way it is also possible to see the development of additional kinetic phases as the light intensity is increased.

Question: Where in both cases most information is found?

The experimenter will note that the light intensity needed for the determination of F_M is in the first case considerably lower than in the second case. The actinic light activates photosynthesis. To reach F_M, a reduced electron transport chain is needed. In the dark-adapted state, where there is a transient block of electron flow on the acceptor side of PS I, this is easier (requires less light) than in the light adapted state, where there is free outflow of electrons at the acceptor side of PS I.

The experimenter may note that (at high time resolution) the information-richness of the fluorescence transients increases as the light intensity is increased. This illustrates the importance of high light intensities for analysis purposes.

With respect to the Light Curves the experimenter will notice that the most interesting part of the light curves is found at rather low light intensities. But the parameters are again determined with strong pulses of light.

References:

Earl HJ, Ennahli S (2004) Estimating photosynthetic electron transport via chlorophyll fluorometry without photosystem II light saturation. Photosynth Res 82: 177-186

Rappaport F, Béal D, Joliot A, Joliot P (2007) On the advantages of using green light to study fluorescence yield changes in leaves. Biochim Biophys Acta 1767: 56-65

Schreiber U, Klughammer C, Schansker G (2019) Rapidly reversible chlorophyll fluorescence quenching induced by pulses of supersaturating light *in vivo*. Photosynth Res 142: 35-50

4.1.9 Attached and detached leaves

If possible, it is always better to measure on leaves attached to a plant. However, to bring a tree into the lab is difficult and it is also not always possible to do measurements *in situ* in the field or the forest, for example. In addition, it should be considered that plants grown in pots may have stressed roots and the soil conditions in a pot are not always those found in nature. Having written this, there are certain physiological consequences induced by cutting a leaf. The most important of these consequences is, no doubt, the closing of the stomata, which strongly reduces gas exchange. This does not mean that there, very quickly, will be no photosynthetic activity anymore, because photorespiration and associated electron transport can also occur in the absence of gas exchange. Stomatal closure can be avoided by cutting the leaf under water and keeping the petiole of the leaf subsequently in water. But even in that case the natural sink-source relationships will disappear.

The important question the experimenter should ask him or herself is, what does he/she want to know and to what extent are the results affected by the conditions under which these measurements are made. It is, e.g., likely that NPQ will be induced at lower light intensities if the stomata are closed. This does not mean, though, that the relationship between lumen pH and NPQ induction has changed. If the experimenter is interested in the light intensity dependence of NPQ-induction, closed stomata form a problem; if, however, the experimenter is interested in the relationship between lumen pH and NPQ closed stomata are not necessarily a problem.

Experimental time: ~1 h

Materials: attached and detached leaves of a C3 plant.

Experiment 1: Make a Light Curve (step length 120 s, 12 light levels) on an attached and detached leaf and look for shifts in the light intensity dependence of the parameters.

Experiment 2: Do a classical Quenching Analysis experiment (actinic light 150-200 µmol photons m^{-2} s^{-1}; and standard settings: delay: 40 s, width: 20 s, length: 15 in the Settings window) on an attached and detached leaf and compare the time dependence of fluorescence induction in both cases (NPQ, qP, F_V'/F_M').

Questions: Is induction faster in detached leaves? Are all three parameters affected in the same way?

References:

Kato MC, Hikosaka K, Hirose T (2002) Leaf discs floated on water are different from intact leaves in photosynthesis and photoinhibition. Photosynth Res 72: 65-70

Potvin C (1985) Effect of leaf detachment on chlorophyll fluorescence during chilling experiments. Plant Physiol 78: 883-886

Raschke K (1970) Stomatal responses to pressure changes and interruptions in the water supply of detached leaves of *Zea mays* L. Plant Physiol 45: 415-423

4.1.10 Memory/Long-term effects

Several processes that can be determined by Chl *a* fluorescence have a memory or to put it differently, they are characterized by long term effects due to the fact that returning to the starting point takes a long time. Such processes are especially relevant when considering needed dark acclimation times of plant species exposed during the day to sun light. The experiment illustrates one such effect, but long-term acclimation and recovery from stress belong to the same category.

Experimental time: ~45 min

Material: a C3 leaf

Experiment: take a leaf of a C3 plant and place it in the magnetic leaf clip. Start the software, in the <Settings> window, set the actinic light to around 400/500 µmol photons m^{-2} s^{-1} (setting 8) and go to the <Chart> window. The moment the software is turned on, the fluorescence is recorded in the <Chart> window. After about 1 min recording, turn the actinic light on by checking <Act. Light> in the bottom panel. Leave the actinic light on for 5 min, then turn it off and 10 min later turn it on for another 5 min. This way the fluorescence is twice induced. Do this for several plants and compare the kinetics of the first and the second illumination period.

The experiment illustrates that if the experimenter is interested in induction kinetics of plants grown outside, it is probably best to make predawn measurements, where the plants are perfectly dark-acclimated and there is a clear reference state. If samples would be taken somewhere in the afternoon it would take hours before all zeaxanthin has been reconverted to violaxanthin again and the induction kinetics will return to their truly dark-acclimated rates.

References:

Demmig-Adams B, Moeller DL, Logan BA, Adams WW (1998) Positive correlation between levels of retained zeaxanthin + antheraxanthin and degree of photoinhibition in shade leaves of *Schefflera arboricola* (Hayata) Merill. Planta 205: 367-374

Horton P, Ruban AV (1992) Regulation of photosystem II. Photosynth Res 34: 375-385

Leong T-Y, Anderson JM (1986) Light quality and irradiance adaptation of the composition and function of pea-thylakoid membranes. Biochim Biophys Acta 850: 57-63

4.1.11 Chloroplast movements

About 20 years ago the genes coding for the blue light receptors of plants (phototropins) were identified. Using knockout mutants it was possible to show that these blue light receptors control chloroplast movements inside the cell. If the blue light intensity is low, chloroplasts concentrate on the abaxial (bottom) side of the palisade parenchyma cells maximizing the absorption cross section. If the blue light intensity is relatively high the chloroplasts move to the lateral sides of the palisade parenchyma cells, minimizing the absorption cross section.

There are two types of JUNIOR PAM instruments: one with only a blue light source and another with white light, but also in this case there is an important blue component. Only PAM instruments that use a red actinic light source will not induce chloroplast movements.

Experimental time: ~1 h

Material: a shade grown and a sun light exposed C3 leaf and depending on the type of JUNIOR PAM a blue light source.

Experiment: Acclimate the leaves to low intensity room light (20-50 µmol photons m^{-2} s^{-1}). Place the leaf in the leaf clip. Start the software and go to the Chart-tab. In this window the fluorescence level is continuously recorded. Determine F_0 and F_M with a saturation pulse by clicking <F_0,F_M>, turn the blue actinic light on (~50 µmol photons m^{-2} s^{-1}) and during 10 min give every 100 s a saturation pulse by clicking <SAT Pulse>.

Question: does the F_M'-value gradually decrease during these 10 min?

References:

Cazzaniga S, Dall'Osto L, Kong S-G, Wada M, Bassi R (2013) Interaction between avoidance of photon absorption, excess energy dissipation and zeaxanthin synthesis against photooxidative stress in *Arabidopsis*. Plant J 76: 568-579

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. Nature 420: 829-832

Pfündel EE, Latouche G, Meister A, Cerovic ZG (2018) Linking chloroplast relocation to different responses of photosynthesis to blue and red radiation in low and high light-acclimated leaves of *Arabidopsis thaliana* (L.). Photosynth Res 137: 105-128

Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) *Arabidopsis* nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci USA 98: 6969-6974

4.1.12 State transitions

There is an extensive literature on 'State' transitions. Classically, State 1 and State 2 are induced by far-red and red light, respectively. These two types of light also form the basis for the discovery of the existence of two photosystems and as well for the fact that the first photosystem in the chain is called PS II and the second PS I. Phenomenologically, state transitions were first described in algae at the end of the nineteen sixties. Ten years later a kinase was described phosphorylating light harvesting complexes II, whose activation is under control of the PQ redox state. State transitions have a much larger effect in green algae and cyanobacteria than in higher plants. The much higher chlororespiratory activity in these organisms (compared to plants) means that the PQ pool will be in a much more reduced state in darkness. Especially cyanobacteria have completely different fluorescence properties in the two states. To keep aquatic samples in state I, it is a good idea to use FR1 background light to keep the PQ-pool in the oxidized state.

Experimental time: 2-3 h

Material: In higher plants state transitions possibly play a role under low light (shade) conditions. Take, therefore, for this experiment a low light/shade grown C3 plant.

Experiment 1: In this experiment, changes in the state of the photosynthetic apparatus are followed by giving every 100 s an SP. In the Settings window set the AL to ~100 µmol photons m^{-2} s^{-1} and the FR to the highest value. Using the Graph tab of the JUNIOR-PAM software light sources can be turned on and off manually. In addition to the parameters that are checked by default, check <qP>, <qN> and <NPQ>. For the first SP use the <F_0,F_M> button

to establish the reference F_0 and F_M values and for the subsequent SPs the <SAT> button. During the first 5 min only the measuring light is turned on. Then, the photosynthetic apparatus is driven to State 2 with 10 min low intensity (~100 µmol photons m^{-2} s^{-1}) blue light of the JUNIOR-PAM. A complication of this protocol is that the actinic light also induces energy quenching (qE), which also affects the F_M'. To allow the qE to relax, on turning off the actinic light, a 5 min delay (only measuring light on) is built into the protocol before the 10 min FR-light illumination is started. The FR-light drives the photosynthetic apparatus to state 1. The FR-light illumination is again followed by 5 min ML. To test if the observed phenomena are fully reversible, a second cycle of 10 min AL, 5 min ML, 10 min FR and 5 min AL is included (Fig. 4-2). At the end of the experiment, click on <Stop Online>, to stop the recording of the fluorescence signal and then click on <Options> and <Export Record> to export the data as a *.csv file. To analyze the data, make figures of Ft, F_M' and qN versus time.

Experiment 2: It is also possible to do the experiment without AL. However, that will only work if the dark-acclimated leaf is in State 2. If it is in State 1, the FR-light should have no further effect. Here, start with 5 min ML, then 10 min FR, 10 min ML, 10 min FR, 10 min ML, 10 min FR, 5 min ML. As in the previous experiment, give every 100 s an SP to monitor changes in F_M' (Fig. 4-2). At the end of the experiment click <Stop Online> to stop recording of the fluorescence signal and then click on <Options> and <Export Record> to export the data as an *.csv file. To analyze the data, make figures of Ft, F_M' and qN versus time.

Questions: 1. On turning on the FR-light the FM' increases and on turning off the FR-light, it decreases. Are the amplitudes of increase and decrease the same? 2. Does the second experiment

work and what does this mean for the dark state of the leaf? 3. How long does it take the drive the system from State 2 to 1 by FR-light and how quickly does it return to state 2 on turning off the FR-light?

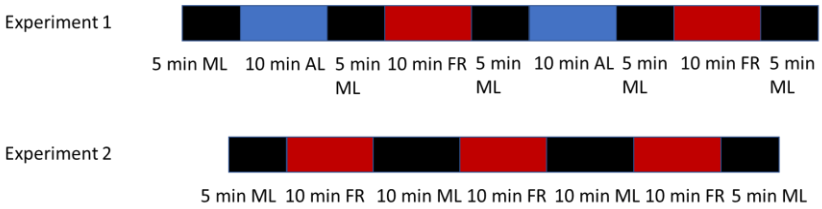

Fig. 4-2: Schematic representation of the experimental protocols for the two State Transition experiments. During the whole experiment every 100 s a saturation pulse is applied.

Supplementary experiment (for instruments with sufficient time resolution): measure an O-I1-I2-P transient (use the appropriate script from the standard set of scripts) on a cyanobacterial suspension with and without FR1 background light.

In the absence of FR1, chlororespiration will keep the PQ-pool of cyanobacteria partially reduced in darkness and drive the photosynthetic system to State 2. FR1 will keep the PQ-pool in the oxidized state in darkness and keep the cyanobacteria in State 1.

In diatoms a slightly different problem driven by chlororespiration plays a role. In those organisms chlororespiratory activity maintains a relatively acid lumen and associated non-photochemical quenching in darkness. NPQ quenches the F_M level and, as a consequence, the F_M measured on dark-adapted diatoms is lower than the F_M measured on diatoms illuminated with FR1 or low intensity background light.

References:

Allen JF, Mullineaux CW (2004) Probing the mechanism of state transitions in oxygenic photosynthesis by chlorophyll fluorescence spectroscopy, kinetics and imaging. In: Papageorgiou GC, Govindjee (eds) Chlorophyll Fluorescence: A Signature of Photosynthesis. Springer, Dordrecht, pp. 447-461

Bonaventura C, Myers J (1969) Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. Biochim Biophys Acta 189: 366-383

Damkjaer JT, Kereïche S, Johnson MP, Kovacs L, Kiss AZ, Boekema EJ, Ruban AV, Horton P, Jansson S (2009) The photosystem II light-harvesting protein Lhcb3 affects the macrostructure of photosystem II and the rate of state transitions in *Arabidopsis*. Plant Cell 21: 3245-3256

Goldschmidt-Clermont M, Bassi R (2015) Sharing light between two photosystems: mechanism of state transitions. Curr Op Plant Biol 25: 71-78

Haldrup A, Jensen PE, Lunde C, Scheller HV (2001) Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Biochem Sci 6: 301-305

Walters RG, Horton P (1991) Resolution of components of non-photochemical chlorophyll fluorescence quenching in barley leaves. Photosynth Res 27: 121-133

The experiments described in the 12 points above were designed to give the experimenter a feeling for different aspects of Chl *a* fluorescence measurements. The sections 4.2-4.5 are about several aspects of the photosynthetic apparatus and its response to stress that can be characterized with Chl a fluorescence.

4.2 Properties of the photosynthetic apparatus

4.2.1 The acceptor side of PS I

Between F_0 and F_M the fluorescence kinetics of angiosperm and gymnosperm leaves are very similar. Beyond F_M, both types of leaf behave quite differently. In angiosperm leaves the fluorescence signal in many cases stays close to F_M for several seconds and subsequently gradually starts to decline. In gymnosperm leaves/needles there is a sharp decrease of the fluorescence signal beyond F_M followed in many cases by secondary kinetics absent from fluorescence curves measured on angiosperm leaves.

Experimental time: 30 min - 1 h

Material: a C3 leaf and a *Ginkgo* leaf or pine needles

Experiment: Place the leaf in the magnetic leaf clip. Start the software and go the the <Chart> window. Illuminate both types of leaves with six 10-s pulses (~1000 µmol photons m^{-2} s^{-1}; setting 10 or 11) spaced 30 s apart and measure 6 times the induced Kautsky kinetics. The actinic light can be turned on and off by checking and unchecking <Act. Light>.

The idea is to induce photosynthesis stepwise in both cases and see the behavior of both types of plants.

Question: describe and compare the Kautsky behavior of both types of leaves/needles.

4.2.2 Quenching below F_0

As demonstrated in the previous experiment, angiosperm leaves are characterized by a slow fluorescence decrease beyond F_M (or P; the PS I acceptor side remains inactive for quite some time) whereas in gymnosperms electrons can leave PS I essentially unhindered within 1 s of illumination. What are the consequences of this difference if photosynthesis is induced at high light intensity?

Experimental time: ~30 min

Material: a C3 leaf and a *Ginkgo* leaf or pine needles

Experiment: Place a dark-acclimated leaf/needles in the magnetic leaf clip. Start the software, go to the <Settings> window and set the actinic light to 1500 µmol photons m^{-2} s^{-1} (setting 12). Then go to the <Chart> window and after about 30 s of recording click <Fo, Fm> to determine the F_0 and F_M values. After another 100 s, turn the actinic light on by checking <Act. Light>. Illuminate the sample for 15 min and during this period compare Ft (actual fluorescence level) with F_0. After 15 min turn the actinic light off and give a saturation pulse 20 s later (click <SAT>) and for the next 15 min click <SAT> every 100 s to follow the relaxation kinetics.

Question: describe the fluorescence kinetics for both types of plants and make a comparison. Is the relative F_M' level after 15 min of darkness different in the two cases?

4.3 Sun/shade acclimation and leaf optical properties

4.3.1 The sidedness of leaves

Fluorescence measurements are almost always made on the top side of the leaf. It is also possible to measure fluorescence from the bottom side of the leaf, but there are several reasons why this is not a good idea.

If we look at the structure of a leaf on going from the top side to the bottom side of the leaf, there are several types of heterogeneity. In classical dicot leaves the top side of the leaf is characterized by palisade parenchyma cells that act as light guides facilitating light penetration deep into the leaf. These cells are bordered on their bottom side by spongiform parenchyma cells that are not so well organized, surrounded in part by intercellular spaces that cause a strong scattering of the light. On its way through the leaf, part of the light is absorbed, and the light intensity decreases. This light gradient is paralleled by a gradient of biochemical properties. Chloroplasts near the top side of the leaf are acclimated to (relatively) high light intensities characterized by more PS II reaction centers with smaller antennae, and a higher capacity of the photosynthetic electron transport chain and Calvin-Benson cycle. The chloroplasts on the bottom side of the leaf are acclimated to low light intensities and are characterized by fewer PS II reaction centers with larger antennae, and lower capacities of the photosynthetic electron transport chains and Calvin-Benson cycle.

Monocots, like grasses, are more symmetrical with a similar cell arrangement on both sides of the leaf (see Fig. 4-3 for a schematic representation of a monocot leaf.

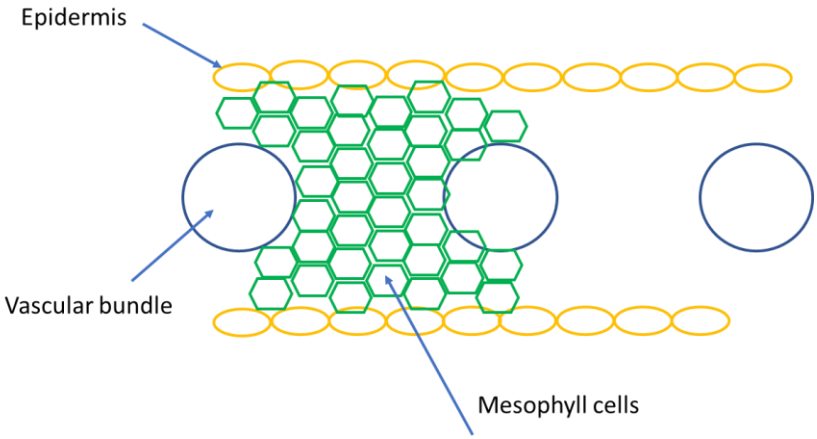

Fig. 4-3: Schematic representation of the structure of a monocot leaf.

Experimental time: ~4 h

Material: dicot (C3), monocot (C3) and C4 leaves

Experiment 1: measure Light Curves (for the JUNIOR-PAM: width: 1:00, Int.: 1, length: 12) from the top and bottom side of one or more dicot and monocot leaves. Pre-illuminate the samples with about 50 µmol photons m^{-2} s^{-1} (setting 2) for 2 min + 2 min of darkness before starting the Light Curve measurements to activate the acceptor side of PS I and the Calvin-Benson cycle.

From the top side the light can probe deeper and probes chloroplasts that are more high light acclimated. From the bottom side the light is strongly scattered and the light probes chloroplasts that are more shade acclimated.

Supplementary experiment: Repeat the same experiment for a C4 plant like maize. C4 plants like maize have a quite different leaf morphology (Krantz anatomy).

Question: Does the electron transport rate (ETR(II)) saturate at lower light intensities and are the maximum values lower for measurements made from the bottom than from the top side? And is it possible to detect the proposed scattering effect for bottom side measurements?

References:

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4.3.2 Shade versus sun leaves

Plants acclimate to the light conditions they are exposed to. Shade and Sun-exposed sites have quite specific effects on plants. Under shade conditions the focus is on light harvesting. This means large PS II antennae, low Calvin-Benson cycle capacity, small PQ pools, low levels of cyt b6f, etc. Sun exposure has the opposite effect: small PS II antennae, high Calvin-Benson cycle capacity, large PQ pool, higher levels of cyt b6f, etc.

Experimental time: ~2 h

Material: sun-exposed and shade leaves of *Hedera helix* (or any other plant of which the two variants are available)

Of *H. helix* there are plants growing in deep shade and plants growing at sun exposed sites. Find a plant with sun and shade versions, make Induction (Quenching Analysis; standard settings: delay: 40 s, width: 20 s, length: 15 in the Settings window; 300-500 µmol photons m^{-2} s^{-1}) and Light Curve measurements (for the JUNIOR-PAM: width: 1:00, Int.: 1) on the top side of the leaf and compare (e.g. rate of NPQ induction, light intensity dependence of NPQ induction, light intensity dependence of qP and ETR(II) as well as maximum ETR(II) rate). Pre-illuminate the samples with about 50 µmol photons m^{-2} s^{-1} (setting 2) for 2 min + 2 min of darkness before starting the Light Curve measurements to activate the acceptor side of PS I and the Calvin-Benson cycle.

On the basis of the properties of sun leaves compared to shade leaves one may expect: 1. That NPQ-induction starts at higher light intensities, but that more NPQ can be induced, that ETR(II) increases more slowly, but that at high light intensities higher ETR(II) values are observed and also that qP (rough indicator for the Q_A redox state) decreases more slowly.

Question: do the experimental results confirm the expectations?

References:

Anderson JM, Chow WS, Goodchild DJ (1988) Thylakoid membrane organization in sun/shade acclimation. Aust J Plant Physiol 15: 11-26

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4.4 Abiotic stress

The abiotic stress related experiments are limited to photoinhibition and heat stress, because these can be induced quickly without the need for special equipment.

4.4.1 Photoinhibition

Plants need light, but in an atmosphere consisting of 20% oxygen they play with fire since chlorophyll molecules, O_2 and excess excitation energy may lead to the generation of oxygen radicals that can destroy (potentially) the photosynthetic apparatus and the photosynthetic membranes. The reaction centers contain carotenoids that can 'quench' the singlet state of chlorophyll molecules, but there is a fine line between photosynthesis and photodamage. PS II has been shown to be more sensitive to photoinhibition than PS I, although the exact mechanism is still a matter of debate. Photoinhibition leads to a loss of variable fluorescence, but in the short term does not affect F_0 (or increases it somewhat). As a consequence, photoinhibition decreases the parameter F_V/F_M and the development of photoinhibition can conveniently be monitored by this parameter. Jones and Kok showed already in 1966 that photoinhibition is determined by the dose. That means that the effect will remain the same if you double the light intensity and halve the treatment time. In this experiment this dose effect will be demonstrated.

Experiment 1: Use a separate light source for the photoinhibition of the leaves. The intensity can be varied by changing the distance between leaf and light source. To avoid heating of the leaf a water filter (a container with a glass bottom filled with water which absorbs far-red light) can be placed between the light source and the sample. 1500 µmol photons m^{-2} s^{-1} x t, 3000 µmol photons m^{-2} s^{-1}

x ½t, 4500 µmol photons m^{-2} s^{-1} x ⅓t, 6000 µmol photons m^{-2} s^{-1} x ¼t and determine the F_V/F_M value 15 min after the end of the photoinhibitory treatment (place the leaves/plants in darkness during these 15 min; dim room light will also do).

The chosen light intensities are not physiological, but the rate of inhibition has to be so high that no interference by repair occurs. Alternatively, the leaves would have to be infiltrated with lincomycin. Is the dose effect observed?

Experiment 2: Run Light Curve measurements (for the JUNIOR-PAM: width: 1:00, Int.: 1) on the created photoinhibited leaves (after 1 h of dark incubation) and run Light Curve measurements on non-inhibited leaves as well. Pre-illuminate the samples with about 50 µmol photons m^{-2} s^{-1} (setting 2) for 2 min + 2 min of darkness before starting the Light Curve measurements to activate the acceptor side of PS I and the Calvin-Benson cycle.

Photoinhibition inhibits (at room temperature) preferentially PSII and PSII is not limiting at low light intensities. Plot the light intensity dependence for ETR for the inhibited and control leaves and plot as well the percentage inhibition of ETR as a function of the light intensity.

Question: PSII limits electron flow at low light intensities, but not at high light intensities. The expectation is, therefore, that the percentage inhibition is higher at low light intensities than at high light intensities. Do the results confirm this expectation?

References:

Jones LW, Kok B (1966) Photoinhibition of chloroplast reactions; I. kinetics and action spectra. Plant Physiology 41: 1037-1043

Lee H-Y, Chow WS, Hong Y-N (1999) Photoinactivation of photosystem II in leaves of *Capsicum annuum*. Physiol Plant 105: 377-384

Schansker G, van Rensen JJS (1999) Performance of active photosystem II centers in photoinhibited pea leaves. Photosynth Res 62: 175-184

Tyystjärvi E, Aro E-M (1996) The rate constant of photoinhibition, measured in lincomycin-treated leaves is directly proportional to light intensity. Proc Natl Acad Sci USA 93: 2213-2218

4.4.2 Heat stress

PS II has on its donor side a manganese cluster which is the catalytic center for water splitting. It catalyzes the reaction $2\ H_2O \rightarrow 4\ H^+ + 4\ e^- + O_2$. The manganese cluster is only stable if the Mn-molecules stay in the Mn^{3+} or Mn^{4+} state. Super-reduction of the Mn-cluster and formation of Mn^{2+} destabilizes the Mn-cluster. Heat stress in darkness leads to a dissociation of the external proteins that limit access of external reductants like ascorbate to the Mn-cluster. A short-term submersion of leaves in a 48-50 °C water bath can destroy all manganese clusters. It, thereby, also destroys the electron generating capacity of all PS II reaction centers, reducing their electron donating capacity to one single stable charge separation.

Light prevents the super-reduction of the Mn-cluster and, therefore, a heat treatment in the presence of light is expected to have a different impact on PS II and on Chl *a* fluorescence measurements.

Experimental time: ~3 h

Material: this type of experiment has in the past been successfully done with pea plants and barley seedlings. In principle any leaf will do, although the exact temperature dependence of inhibition may vary between species. Further, a thermometer to determine the temperature of the water bath, a heating element to bring the water bath to the selected temperature, a beaker with water, large enough to submerge for example the seedlings in. A somewhat larger water bath will also keep the water temperature stable for a longer time. While heating the water bath, the water has to be mixed regularly or continuously to homogenize the water temperature. It is also possible to heat water in a water cooker, add it to

a beaker and add cold water under mixing until the target temperature is reached.

Experiment 1: Michel Havaux (1996) did experiments that indicated that heat stress stimulates the non-photochemical reduction of the PQ-pool (e.g. via cyclic electron transport around PS I). In some plant species this effect is quite spectacular, e.g. in *Geranium pratense* (meadow crane's-bill or meadow geranium), in Western-Europe a quite common plant. Submerge, if possible, a *Geranium pratense* leaf for 40 s in a 49 °C water bath in darkness. Subsequently, wait 30 min before doing the experiments. Start the software, set the actinic light intensity to approx. 420 µmol photons m^{-2} s^{-1} and go to the Chart tab. The measuring light box should be checked and after about 30 s, turn on the actinic light by checking the box. After 5 min of illumination turn off the actinic light (uncheck the box) and continue the measurement for 10 min with only ML. Then turn the AL on again for another 5 min and after turning the AL off again monitor the fluorescence for another 5 min. Then, stop the online measurement and export the data to an *.csv file. Do the same experiment with an untreated *Geranium* leaf.

Question: what happens on turning off the actinic light. What is the effect of the heat stress treatment on the AL-off fluorescence kinetics?

Experiment 2: comparison between a 20-40 s submersion in a 48-50 °C water bath of samples in darkness and in the presence of 50-100 µmol photons m^{-2} s^{-1}. In the case of barley seedlings, it is possible to submerge a pot with seedlings upside-down in a water bath of the selected temperature. Leave the treated leaves or plants 30 min/1 h in darkness before starting with the measurements. Run a Quenching Analysis (standard settings: delay: 40 s,

width: 20 s, length: 15 in the Settings window, ~500 µmol photons m^{-2} s^{-1}) on a heated and a control leaf.

Experiment 3 (for instruments with sufficient time resolution): Do the treatment at 38-40-42-44-46-48-50 °C and look at the effects of the temperature on the kinetics of SPs (e.g. ~5000 µmol photons m^{-2} s^{-1}). Then, turn on the actinic light (~1000 µmol photons m^{-2} s^{-1}) and record the fluorescence kinetics during 3 min at each temperature.

Supplementary experiment: Heat stress has very specific effects on O-I1-I2-P transients. Measure, if possible, such transients on the leaves of *Experiment 2*.

References:

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4.5 Field experiments

It is not difficult to take a fluorometer outside and randomly measure leaves. The problem is how to interpret the measurements? In the measurements discussed above, each time, the leaves were dark adapted. Dark-adaptation times of less than 1 hour have, in general, two goals: the re-oxidation of the photosynthetic electron transport chain (Q_A, PQ pool, Fd pool, decay of the S-states of the oxygen evolving complex to S1) and the inactivation of FNR and the Calvin-Benson cycle. Other processes like the repair of photoinhibitory damage to PS II and the epoxidation of zeaxanthin to violaxanthin take considerably more time. The F_0 and F_M values of such dark-adapted leaves represent references values that help us to interpret the measurements.

If we go into the field, it is often not possible to dark adapt a large number of leaves for an hour. For remote sensing measurements this is even more of an issue. In the literature two main solutions can be found that both demand a bit of sacrifice. For PAM type measurements, the most used method is the predawn measurement to obtain reference values. As a rule of thumb, 20-25 F_0 and F_M measurements will give a meaningful average for these parameters and also an idea about the variability between measurements. For subsequent measurements during the day these reference values can then be used. Parameters like qP and F_V'/F_M' can be determined without knowledge of F_0 and F_M, but also in that case it is useful to know what the kinetics were in the dark-adapted state.

The alternative approach, more frequently applied in the case of short direct fluorescence measurements, is to make measurements late in the evening, where leaves have been in darkness for

several hours. Measuring in semi-darkness has as an additional advantage that no further dark adaptation is needed.

A completely different approach is to measure Light Curves on leaves as is. Just apply a leaf clip and start measuring. In order to catch the state of the leaf, certain eco-physiologists have proposed to keep the steps of the Light Curve as short as possible, e.g. 10 s per light intensity. In this way the experimenters hoped to minimize the effect of the light protocol and to catch the state of the leaf at the moment the measurement was started.

Suggestions for experiments:

4.5.1 Canopy variability around a big tree

Find a big tree and measure 2-3 leaves from the North, East, South and West side of the tree. These leaves will differ in terms of their exposure to the sun during the day. Use aluminum foil to dark adapt leaves but plan the experiment in such a way that the time of dark adaptation for all leaves is approximately the same.

Measure on each leaf a light curve (e.g. 60 s per light step), give in each case as pre-treatment 2 min ~100 µmol photons m^{-2} s^{-1} + 2 min darkness to activate photosynthesis in each case in the same way.

4.5.2 Canopy gradients in a maize or wheat field

The same experiment, but now test different leaf stories within a maize or wheat field. Go far enough in the field to avoid interference of light coming from the side of the field.

4.5.3 Drought stress

There are different forms of drought stress. One form can occur during summer, where plants start out with sufficient water, but

gradually get drought stressed when no further rain falls, and the soil gradually dries out. Another form occurs when plants have access to water, but not enough for optimal growth. Find a naturally drying out site, identify the plant species that grow there, measure them (e.g. a Light Curve, 60 s light steps, preceded by 2 min ~100 µmol photons m^{-1} s^{-1} + 2 min darkness) and try to determine which plant species copes best with the limited water supply. Also judge the plants visually for wilting and necrosis effects. Again, use aluminum foil for the dark adaptation of the leaves to be measured.

If there are C4 plants among the species measured, are they the winners, because C4 photosynthesis can work efficiently at lower CO_2 concentrations and, therefore, C4 plants can keep their stomata more closed, limiting transpiration losses?

What are the best parameters to determine how well the plants cope with drought?

5 Selected publications and reviews on Chl *a* fluorescence

5.1 Chl *a* fluorescence reviews

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Kalaji HM, Schansker G, Ladle RJ, Goltsev V, Bosa K, Allakhverdiev SI, Brestic M, Bussotti F, Calatayud A, Dąbrowski P, Elsheery NI, Ferroni L, Guidi L, Hogewoning SW, Jajoo A, Misra AN, Nebauer SG, Pancaldi S, Penella C, Poli DB, Pollastrini M, Romanowska-Duda ZB, Rutkowska B, Serôdio J, Suresh K, Szulc W, Tambussi E, Yanniccari M, Zivcak M (2014) Frequently asked questions about *in vivo* chlorophyll fluorescence: practical issues. Photosynth Res 122: 121-158

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Murchie EH, Lawson T (2013) Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J Exp Bot 64: 3983-3998

5.2 PAM-method

Schreiber U (2004) Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou GC, Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, Dordrecht, pp 279-319

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5.3 Reviews non-photochemical quenching and xanthophyll cycle

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Horton P, Johnson MP, Perez-Bueno ML, Kiss AZ, Ruban AV (2008) Photosynthetic acclimation: does the dynamic structure and macro-organisation of photosystem II in higher plant grana membranes regulate light harvesting state? FEBS J 275: 1069-1079

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Müller P, Li X-P, Niyogi KK (2001) Non-photochemical quenching; A response to excess light energy. Plant Physiol 125: 1558-1566

5.4 Photochemical and non-photochemical quenching

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Klughammer C, Schreiber U (2008) Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the Saturation Pulse method. PAM Application Notes 1: 27-35

Laisk A, Oja V, Rasulov B, Eichelmann H, Sumberg A (1997) Quantum yields and rate constants of photochemical and nonphotochemical excitation quenching. Plant Physiol 115: 803-815

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5.5 Components of non-photochemical quenching

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Walters RG, Horton P (1991) Resolution of components of non-photochemical chlorophyll fluorescence quenching in barley leaves. Photosynth Res 27: 121-133

5.6 Nomenclature

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5.7 F690/F735

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5.8 Field measurements

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5.9 PSI contribution to F_0

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5.10 ETR

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5.11 Light curves and rapid light curves

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5.12 Fluorescence measurements on algae

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5.13 Diatoms

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5.14 Corals

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5.15 Special aspects of Chl *a* fluorescence

Klughammer C, Schreiber U (2015) Apparent PS II absorption cross-section and estimation of mean PAR in optically thin and dense suspensions of *Chlorella*. Photosynth Res 123: 77-92

Lavergne J, Leci E (1993) Properties of inactive photosystem II centers. Photosynth Res 35: 323-343

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6 Appendix: Example experiments

6.1 Experiment 4.2.2: Pulse length, pulse intensity

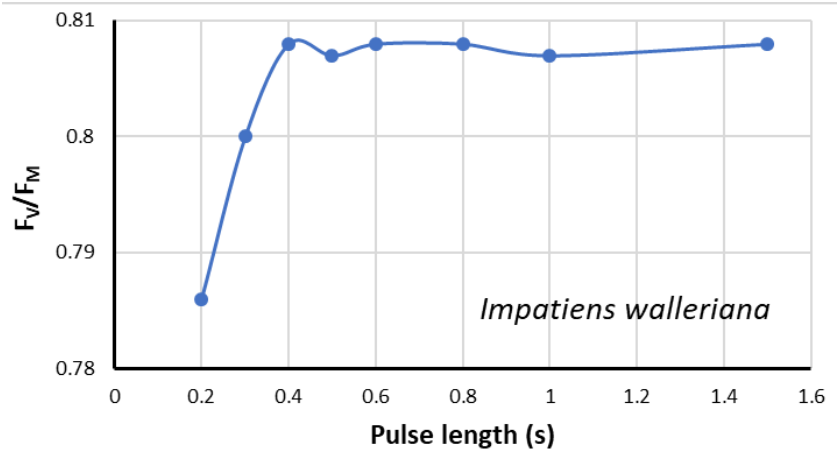


Fig. 6-1: F_V/F_M versus pulse length.

The optimal pulse is long enough to reach F_M, but not so long that the fluorescence level starts to go down again. This last point can be checked on the SAT-Chart tab. If the fluorescence at the end of the pulse goes down again it means that the pulse starts to induce non-photochemical quenching. In the case of leaves a pulse length of at least 0.4 s is needed.

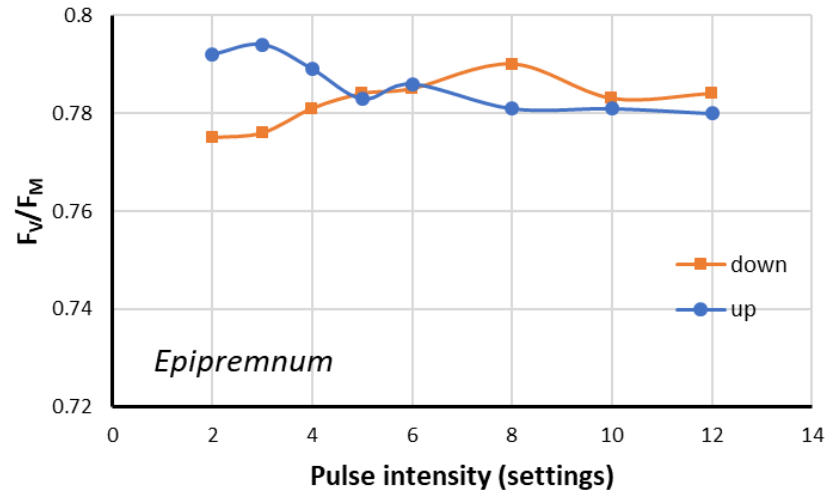
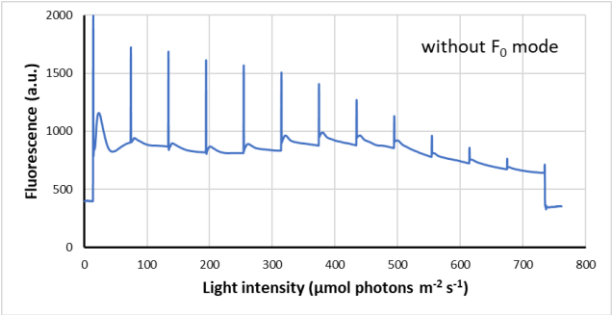
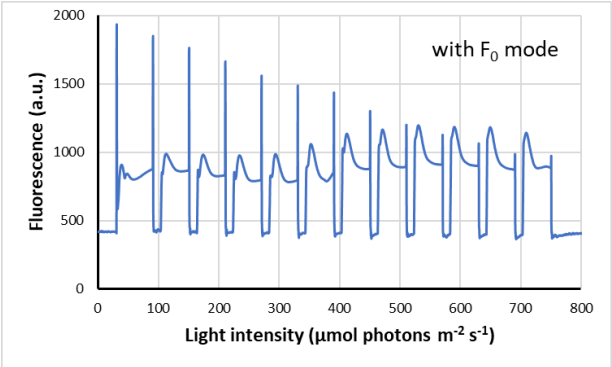


Fig. 6-2: F_V/F_M versus pulse intensity.

Series of 0.5 s saturation pulses spaced 5 min apart applied to an *Epipremnum* leaf. The pulse intensity was either stepwise increased or decreased. A small order effect is observed, but the main message of the figure is that relatively low pulse light intensities are sufficient to induce F_M in a dark-acclimated leaf.

6.2 Experiment: 4.1.6 F_0'-calculation





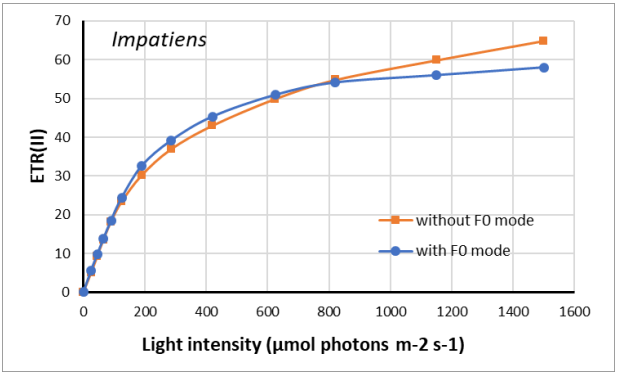


Fig. 6-3: F_0' mode.

Comparison of light curve measurements made with and without F_0 mode. Comparing the top two figures demonstrates that the introduction of FR-pulses to allow determination of the F_0' values changes the fluorescence kinetics considerably. However, looking at the ETR data in the bottom panel demonstrates as well, that the effect on at least ETR, especially at the lower light intensities, is quite limited. At the highest light intensities, a clear saturation is observed when the F_0 mode is applied, which is missing when the F_0 mode was turned off.

6.3 Experiment: 4.1.10 Memory/Long-term effects

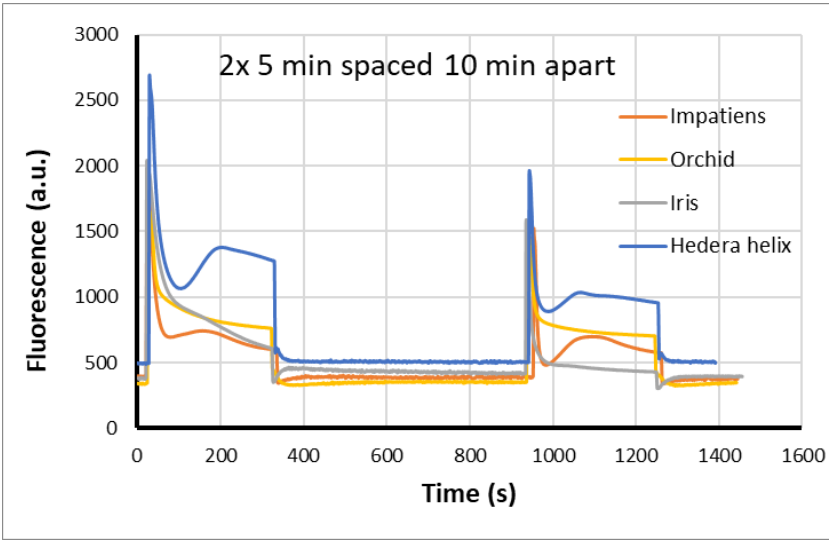


Fig. 6-4: Memory effect.

A memory effect means that the kinetics of a photosynthetic process are affected by what happened before. In the case of stress that can be something that happened days or more beforehand. Here, a short time interval (10 min) is used for this purpose. Several aspects of the induction kinetics are different the second time. In addition, the induction kinetics are quite species dependent.

6.4 Experiment: 4.1.12 State transitions

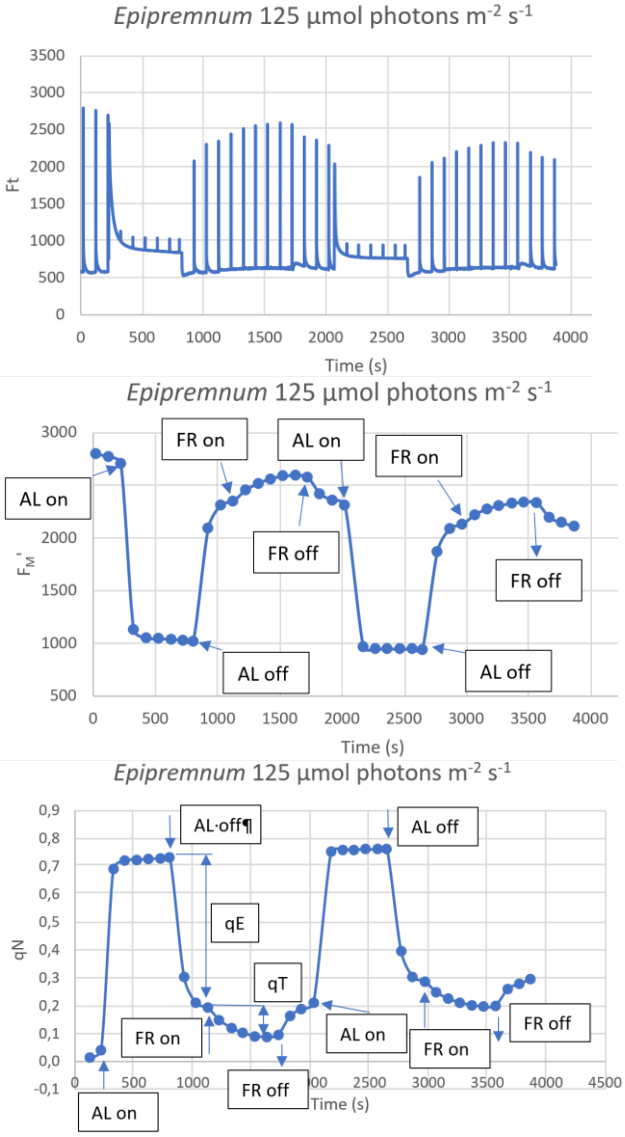


Fig. 6-5: State transitions under actinic light.

State transition effects can be made visible both in the presence and absence of actinic light. Five min of darkness seems to be enough to allow the near complete relaxation of energy quenching.

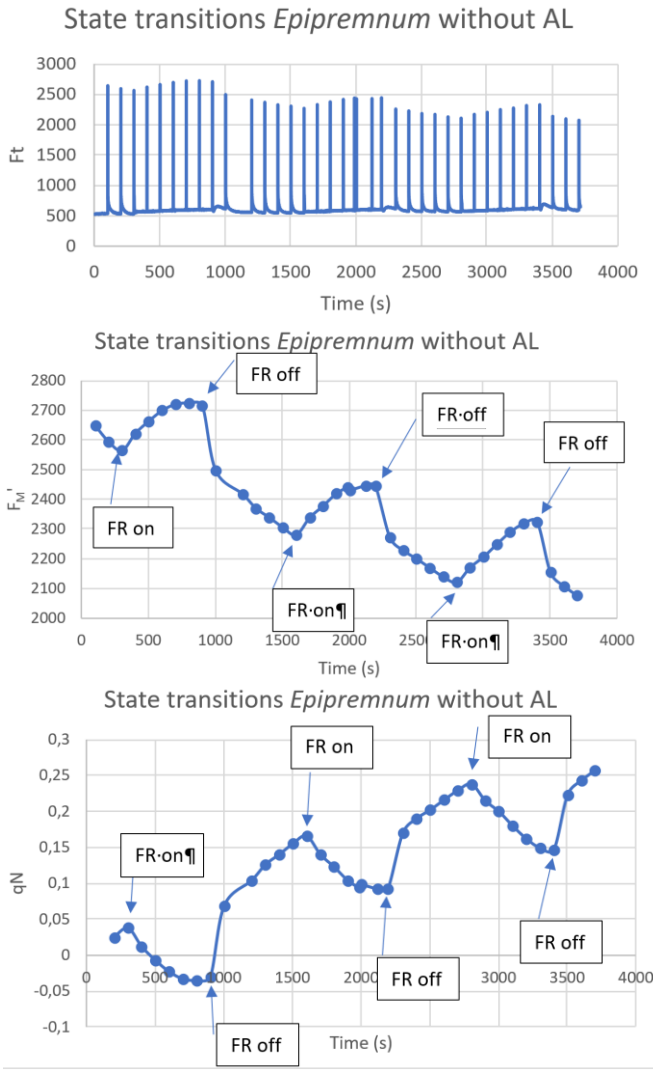


Fig. 6-6: State transitions without actinic light.

State transition effects can be made visible both in the presence and absence of actinic light. Five min of darkness seem to be enough to allow the near complete relaxation of energy quenching.

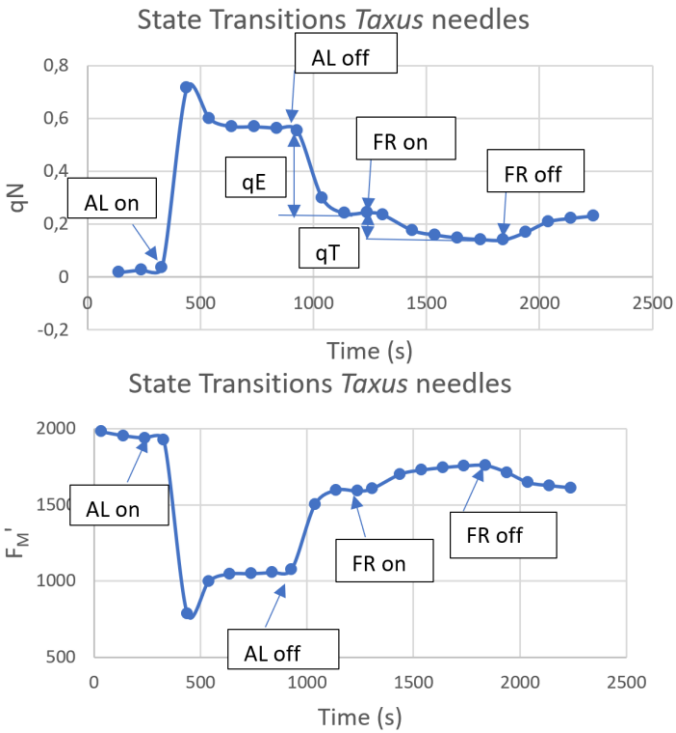


Fig. 6-7: State transitions in *Taxus* needles.

Experiment to show that the state transition approach also works for gymnosperms like *Taxus baccata*. Following a 5 min period of darkness, a 10 min illumination with 90 µmol photons m^{-2} s^{-1} was used to make certain that State 2 was induced. The inverse State 2 to State 1 transition was subsequently induced with 10 min FR light. The state-transition-induced effect on qN (i.e. qT) can then be quantified as the FR-induced change in qN. For this purpose, it is also possible to work with the parameter NPQ.

6.5 Experiment: 4.2.1 The acceptor side of PS I

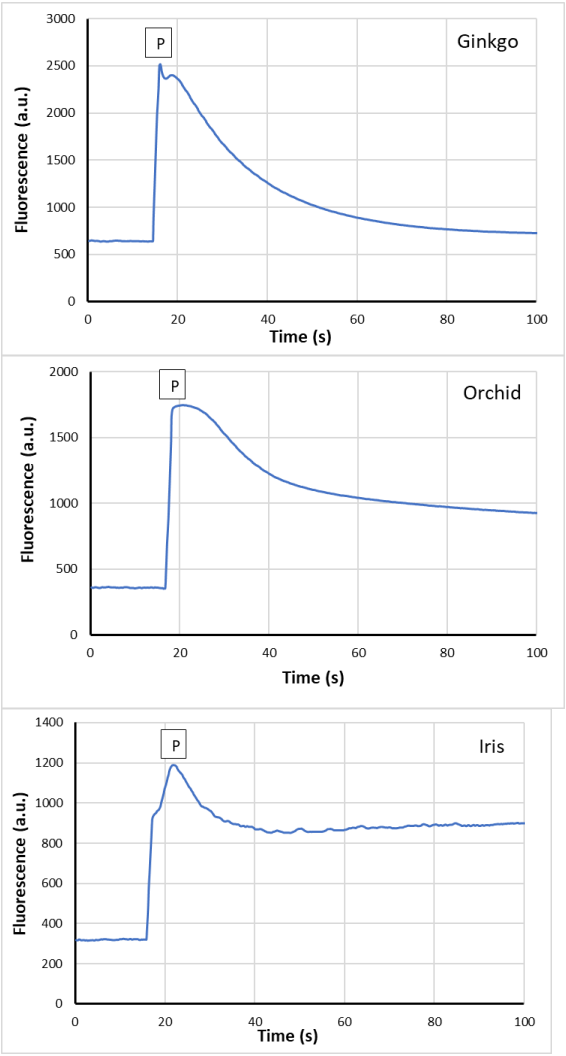


Fig. 6-8: PS I acceptor side.

Kautsky curves at 420 µmol photons m^{-2} s^{-1}. The fluorescence kinetics up to P are largely determined by the reduction kinetics of the electron transport chain. The fluorescence decay beyond P depends much more on the outflow of electrons on the acceptor side of PS I. The *Ginkgo biloba* kinetics are characterized by a fast fluorescence decrease beyond P, followed by a dip and a slow subsequent decrease. The orchid is characterized by a considerable delay beyond P before the fluorescence level starts to decline. Iris leaves have in some respects in-between characteristics.

6.6 Experiment: 4.2.2 Quenching below F_0

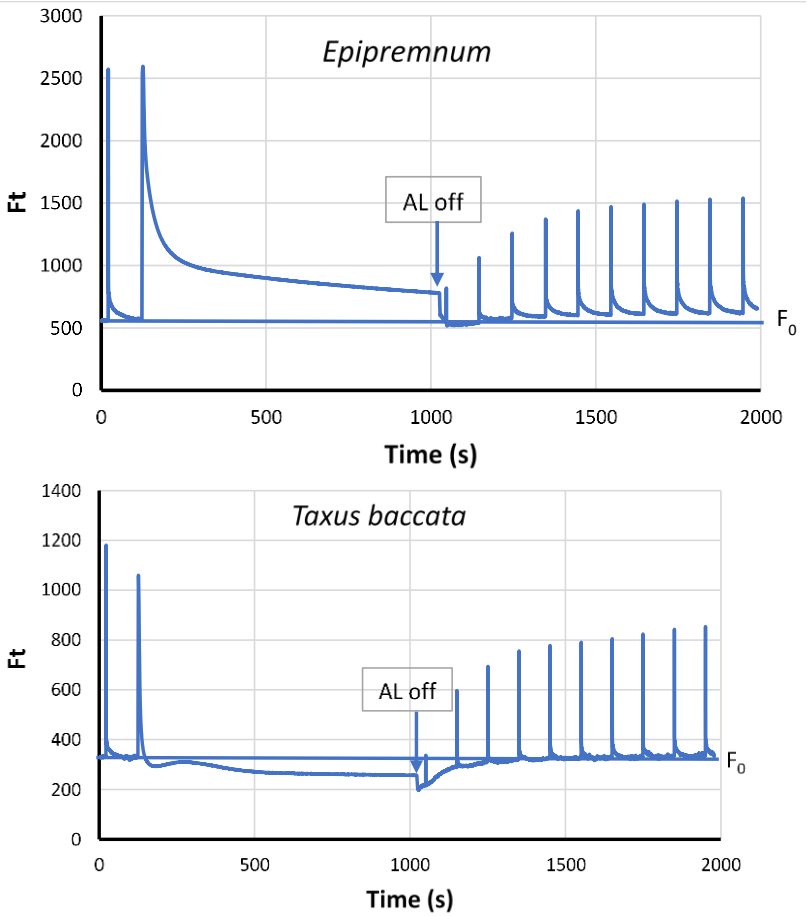


Fig. 6-9: Strong F_0 quenching.

On applying a high light intensity (1500 µmol photons m^{-2} s^{-1}) to dark-adapted angiosperm leaves like *Epipremnum* leaves Ft, in general, stays above F_0. As shown in the bottom panel, the same treatment applied to needles of the gymnosperm *Taxus baccata* quenches Ft below F_0.

6.7 Experiment: 4.3.1 The sidedness of leaves

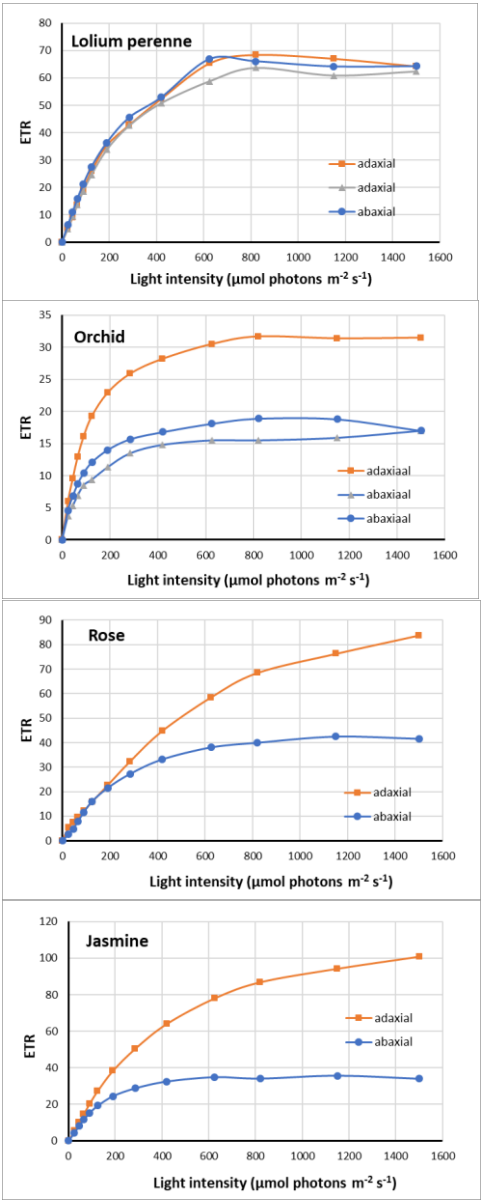


Fig. 6-10: The sidedness of leaves.

Lolium perenne (ryegrass) and Orchids are monocots, whereas rose and jasmine are dicots. For ryegrass the light intensity dependence of ETR of the two sides of the leaves is the same. The measured Orchid is also a monocot, but there the bottom side of the leaf has a considerably lower maximum ETR value than the top side of the leaf. This may have to do with the thickness of the orchid leaves and the fact that the bottom side of these leaves is exposed to considerably less sun light. The dicots rose and jasmine show differences in ETR between both sides of the leaf typical for dicot leaves.

6.8 Experiment: 4.3.2 Shade versus sun leaves

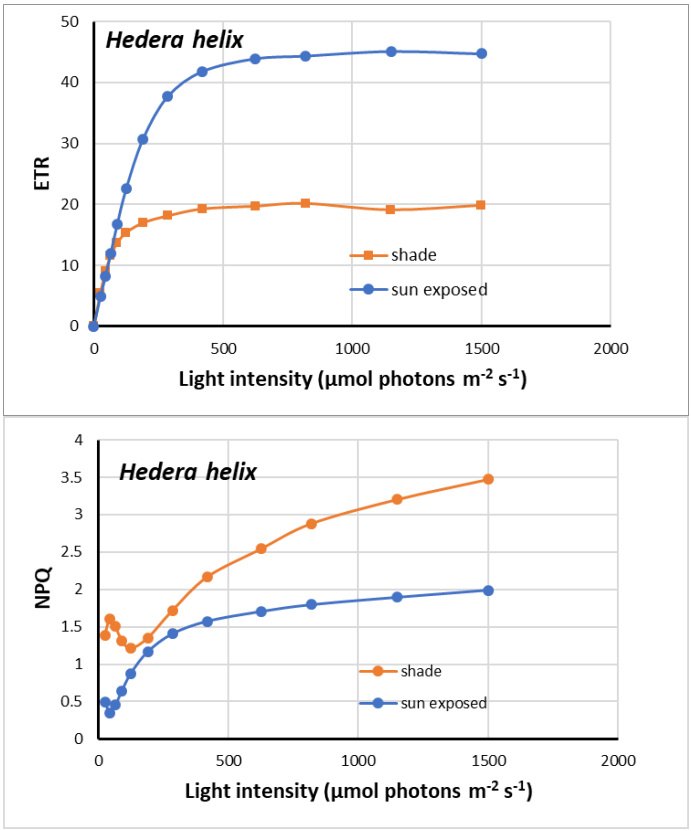


Fig. 6-11: Sun versus shade leaves.

When comparing shade and sun exposed leaves, there are a few typical differences. Sun exposed leaves have a much higher Calvin-Benson cycle capacity and this translates into much higher maximum ETR values. At low light intensity the ETR-values of the shade leaves are slightly higher than the ETR-values of the sun exposed leaves. This is due to the fact that shade leaves have in general larger PS II antennae. The lower electron transport capacity in shade leaves also translates into higher NPQ values.

6.9 Experiment: 4.4.1 Photoinhibition

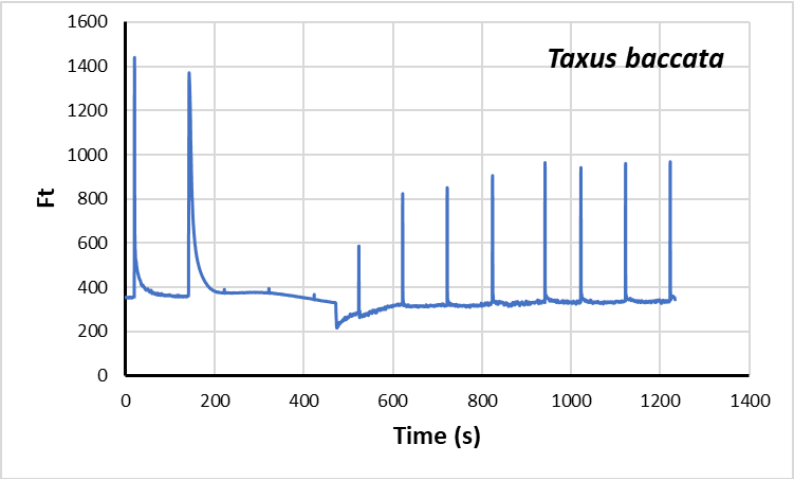


Fig. 6-12: Fluorescence recovery.

Taxus baccata needles exposed to 5 min 1500 µmol photons m^{-2} s^{-1}. The recovery phase indicates that this represents a (mild) photoinhibitory treatment.

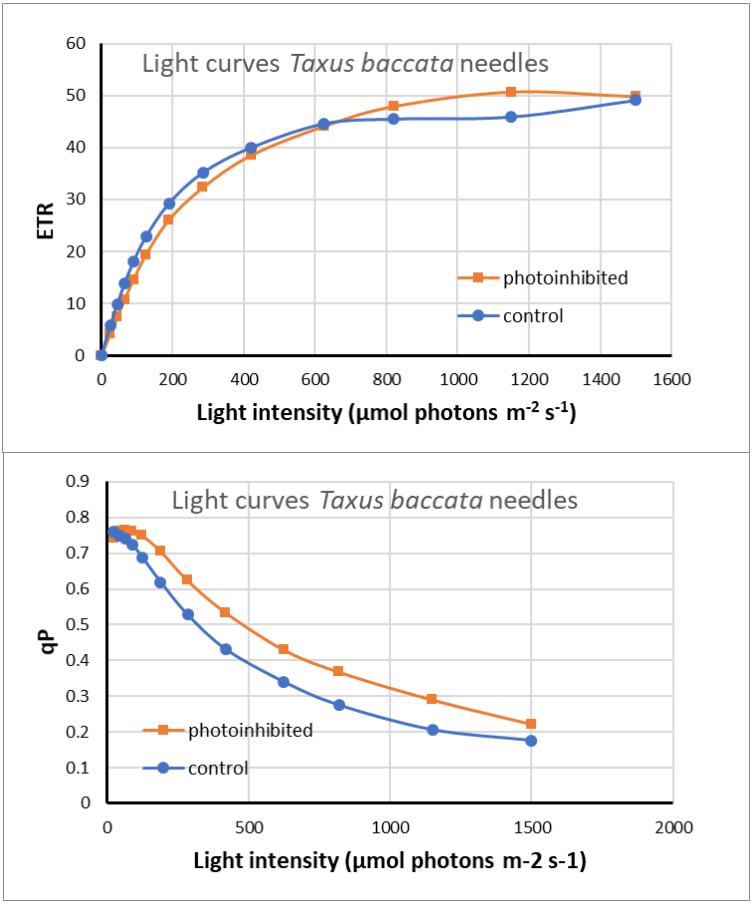


Fig. 6-13: ETR and q_P versus light intensity.

The two figures show two aspects of a photoinhibited sample. The ETR data show that (mild) photoinhibition has some effect at low light intensities, where PS II is limiting, but not at high light intensities. And the qP data show that the still active PS II reaction centers have to work harder to maintain the same electron transport rate and as a consequence the accumulation of Q_A^- (lower q_P) occurs at higher light intensities.

6.10 Experiment: 4.4.2 Heat stress

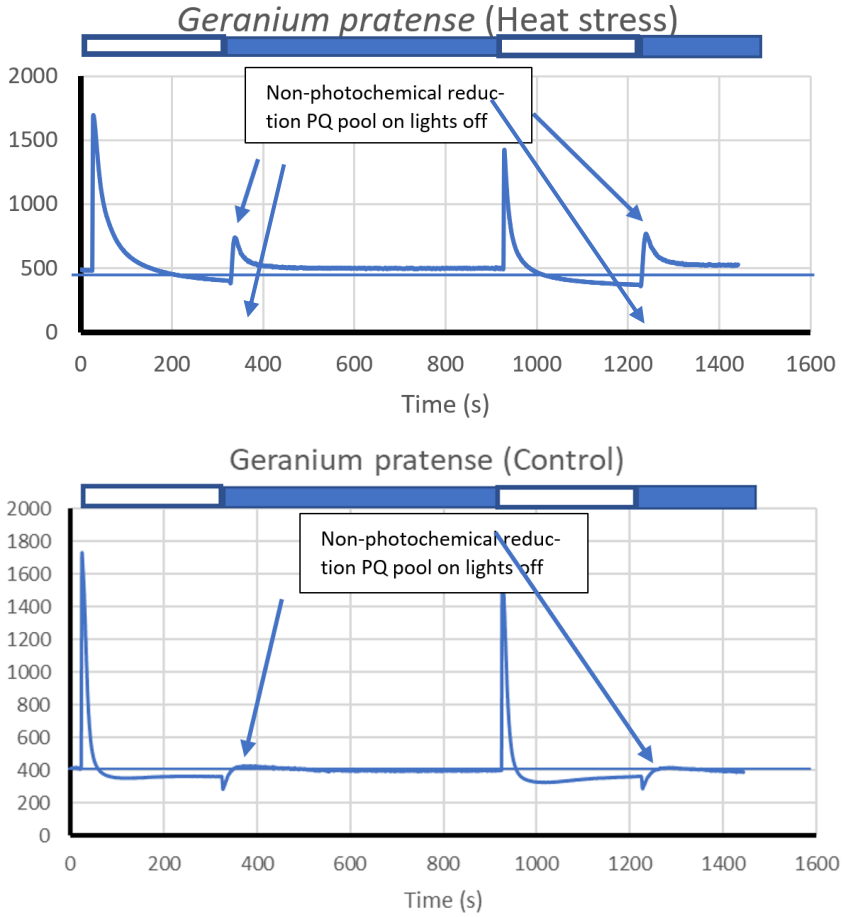


Fig. 6-14: Heat stress Geranium.

Heat stress is thought to stimulate non-photochemical reduction of the plastoquinone pool. However, this effect is quite plant species dependent and it is strong in *Geranium pratense* (meadow crane's-bill).

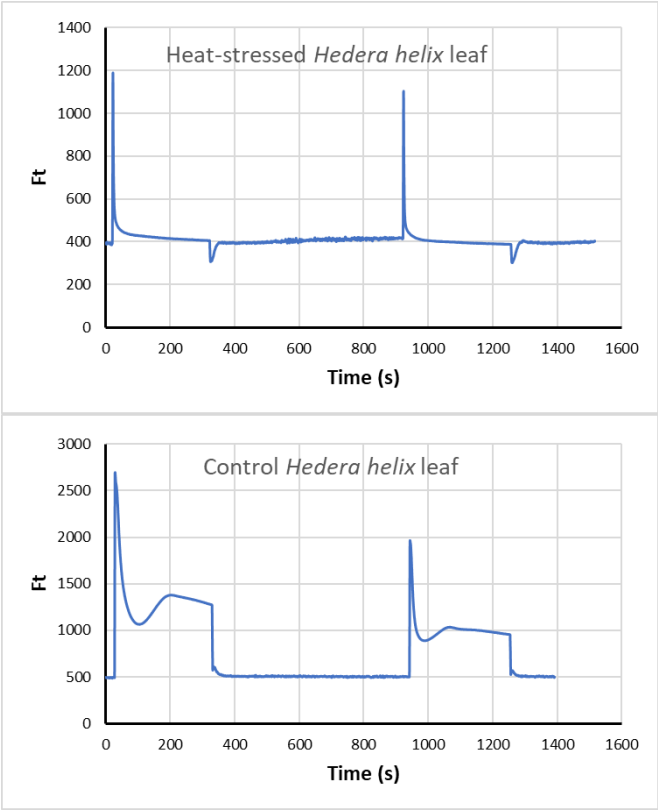


Fig. 6-15: Heat stress Hedera.

Comparison of a heat-stressed *Hedera helix* leaf with a control leaf. In both cases the leaves were illuminated twice with 5 min 420 µmol photons m^{-2} s^{-1} spaced 10 min apart. Control *Hedera* leaves show very little non-photochemical reduction of the PQ pool following lights off (no transient increase of the measured F_0 value). Heat stress stimulates this process somewhat, but the effect is by far not as spectacular as that observed for meadow crane's-bill. The comparison shows that in the heat stressed leaves (most manganese clusters destroyed) there is twice a single narrow fluorescence peak. All secondary kinetics observed for the control leaves are missing because the damaged PS II reaction centers cannot keep up with the outflow of electrons out of the electron transport chain.

7 List of figures