

## The fluorescence transient as a tool to characterize and screen photosynthetic samples

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### Introduction

Photosynthesis is the process by which plants, algae, cyanobacteria and photosynthetic bacteria convert radiant energy into a chemically stable form. The pathway of this energy transduction is complex, involving several physical and chemical mechanisms and many components. The process is initiated when light is absorbed by the antenna molecules within the photosynthetic membrane. The absorbed energy is transferred as excitation energy and is either trapped at a reaction centre and used to do chemically useful work, or dissipated mainly as heat and less as emitted radiation – fluorescence. The features of the emitted fluorescence are basically determined by the absorbing pigments, the excitation energy transfer, and the nature and orientation of the fluorescing pigments. However, fluorescence is also affected by the redox state of the reaction centers and of the donors and acceptors of PSII, and is moreover sensitive to a wide variety of photosynthetic events, e.g., proton translocation, thylakoid stacking and unstacking, ionic strength, and the midpoint potential of *cyt b-559*, to name a few. Although the effect of each factor on fluorescence is often indirect and they are not easily quantified and distinguished from one another, fluorescence measurements have been successfully used to monitor and characterize a wide variety of photosynthetic events.

The first significant realization of the relationship between primary reactions of photosynthesis and Chl *a* fluorescence came from Kautsky and Hirsh (1931). They were the first to report that, upon illumination of a dark adapted photosynthetic sample, the Chl *a* fluorescence emission is not constant but exhibits a fast rise to a maximum followed by a decline to reach finally, in a range of some minutes, a steady level. They postulated that the rising phase of this transient, found to be unaffected by temperature changes (up to 30°C) and the presence of poison (potassium cyanide), reflects the primary reactions of photosynthesis. They further showed that the declining phase of the fluorescence transient is correlated with an increase in the CO<sub>2</sub> assimilation.

Since this first report, which is a landmark in the history of photosynthesis research, our knowledge on the relationship between primary reactions of photosynthesis and Chl *a* fluorescence has increased tremendously, as this aspect attracted the interest of many research groups due to its significance for basic biophysical research as well as applied research. During these decades the investigations became more and more thorough utilizing also the advancement in instrumentation, and the number of publications on this topic has rapidly increased. For reviews on Chl *a* fluorescence, readers may consult, among others, the following: Rabinowitch (1951), Wassink (1951),

Govindjee *et al.* (1967), Goedheer (1972), Papageorgiou (1975), Lavorel and Etienne (1977), Butler (1978), Govindjee and Jursinic (1979), several chapters in Govindjee *et al.* (1986), Govindjee and Wasielewski (1989), several chapters in Lichtenthaler (1988), Holzwarth (1991), Karukstis (1991), Krause and Weis (1991), Dau (1994a,b), Govindjee (1995), Joshi and Mohanty (1995), Schreiber *et al.* (1995), Sauer and Debreczeny (1996), Owens (1996) and Strasser *et al.* (1997).

This chapter is not a general review on Chl *a* fluorescence and is not written for specialists in the field of fluorescence. It rather aims to give a general introduction to non-specialists about the Chl *a* fluorescence kinetics and how their analysis and interpretation can provide information about the photosynthetic capacity and the vitality of the plant material. Furthermore, this chapter describes analytically a real screening procedure by which the fluorescence transient, easily measured with handy, commercially available, instruments, is analysed providing a description of the dynamic capacities of the photosynthetic sample. This procedure satisfies the demand for rapid, non-invasive screening tests, and can be thus easily applied to approach also questions of commercial or economical interest concerning the physiological condition of plants.

### The fluorescence transient

A typical fluorescence transient, exhibited upon illumination of a dark-adapted photosynthetic sample by saturating light, is shown in Figure 25.1. The same transient is presented here on different time scales. It is clearly demonstrated that on a logarithmic time scale the full transient from 50  $\mu$ s to many minutes can be presented in one graph, revealing both the fast polyphasic rise from  $F_0$  to  $F_p$  (where  $F_p = F_M$  under saturating excitation light) and the subsequent slower decline from  $F_p$  to a steady state  $F_s$ . The fluorometer here used, a PEA (Plant Efficiency Analyser from Hansatech), has a high time resolution (10  $\mu$ s) and a data acquisition capacity over several orders of magnitude (Strasser *et al.*, 1995). With an instrument of lower time resolution the shape of the fast rise of this transient would not reveal the steps O-J-I-P so clearly.

Not only dark-adapted samples upon illumination, but also light-adapted samples, upon any change of the quality (Strasser, 1985) or the intensity (Srivastava *et al.*, 1995) of the light they are exposed to, exhibit a fluorescence transient which levels off at a new steady state. More generally, under natural *in vivo* conditions, the fluorescence behaviour of any photosynthetic system changes continuously following its adaptation to a perpetually changing environment (Srivastava *et al.*, 1995).

Based on several observations, we can conclude that, at a given moment, the shape of the fluorescence transient of any sample is determined by the physiological state of the sample at that moment and the physical and chemical environmental conditions around the sample. It must also be pointed out that the actual physiological state of a sample at a given moment is a function of all the states the sample went through in the past (Strasser, 1985; Krüger *et al.*, 1997).

### Scenario for a practical application of the fluorescence transient

Based on the general conclusions stated above, the fluorescence transient becomes a potential tool for several basic and applied projects. Four basic groups of questions that such projects could aim to tackle are presented in Table 25.1. Examples for each group are:

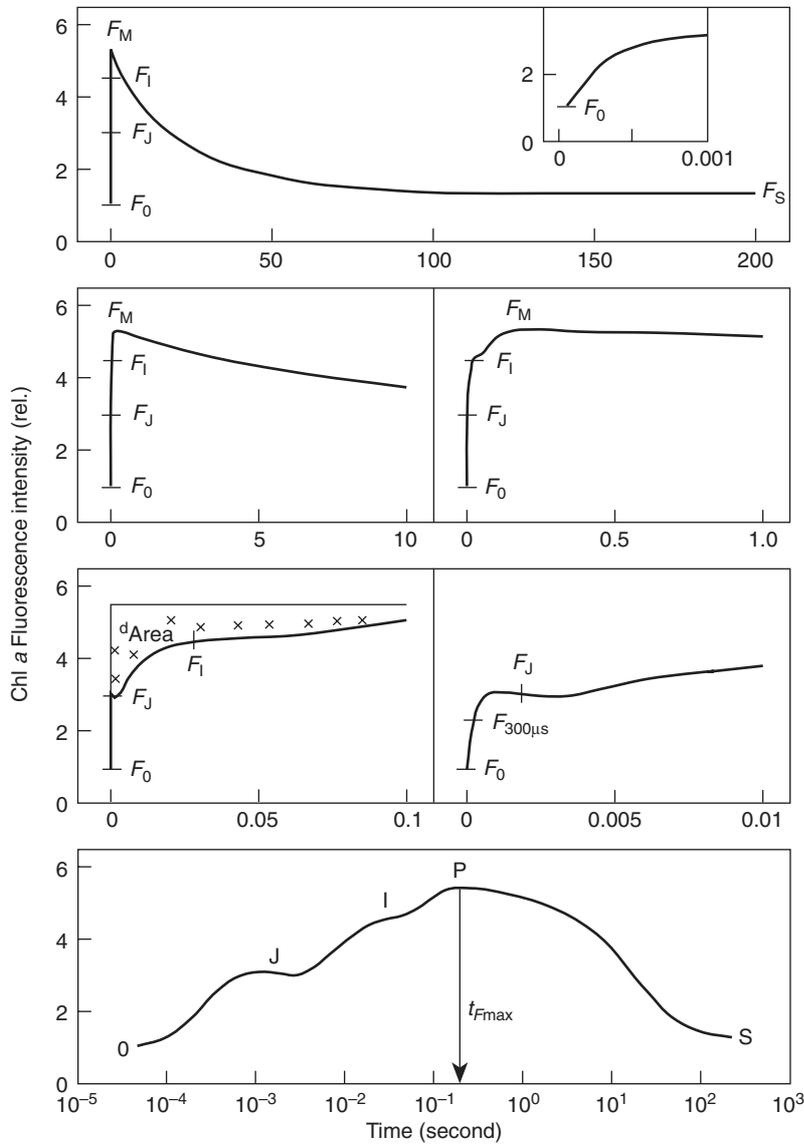


Figure 25.1 A typical fluorescence transient exhibited upon illumination of a dark-adapted photosynthetic sample by saturating light. The transient is presented on different time scales.

- I A well-defined plant material is used under well-defined physical conditions to analyse its response to chemical changes in the environment (chemical stress). Several experiments can be designed in the laboratory, using, e.g., inhibitors, fertilizers, gases like  $\text{CO}_2$ ,  $\text{O}_2$ ,  $\text{O}_3$  or any other chemical stressor.
- II A well-defined plant material is used to analyse its response to changes of physical parameters of the environment (physical stress), e.g., light quality and intensity,

Table 25.1 Four basic groups of questions that can be tackled through a practical application of the fluorescence transient.

Plants	Test conditions (1)	Test conditions (2)	Test signals
The physical and chemical conditions during the culture of the samples	The physical conditions in the environment during the fluorescence test	The chemical conditions in the environment during the fluorescence test	Fluorescence transient as a tool to analyse the following groups of questions
constant	constant	variable	I
constant	variable	constant	II
constant	variable	variable	III
variable	constant	constant	IV

temperature, etc. (Srivastava and Strasser, 1996; Srivastava *et al.*, 1997).

III A well-defined plant material is used to analyse its response to a combination of chemical and physical stress. The synergism and antagonism of the physical and chemical co-stress can be studied (Ouzounidou *et al.*, 1997).

IV Biosensing: under well-defined experimental conditions fluorescence tests are elaborated (a) to describe a system (plant or ecosystem) in terms of, e.g., (i) vitality, (ii) productivity, (iii) sensitivity and resistance to stress; (b) to study the structure – function relationship in transgenic plants; (c) to investigate the ecodynamics of complex systems, like trees, horticulture, forests and even whole ecosystems (Van Rensburg *et al.*, 1996; Srivastava and Strasser, 1997).

The above examples give only an indication of the wide diversity in addressing possible questions and designing the appropriate experiments. Further applications of interest can be enumerated:

- Testing of productivity in agriculture as a function of:
  - style of culture (sustainable agriculture etc.)
  - regulator (herbicides, pesticides, hormones etc.)
  - selection of cultivar (transgenic etc.)
  - drought, heat, cold, light, salt stress etc.
- Testing of the behaviour of a commercialized product. Freshness, taste, colour and consistency of vegetables, flowers and fruits as a function of storage and home conditions; moreover, the decision for the optimal moment for such products to be put on the shelves in the supermarket can be made by utilizing the fluorescence techniques.
- Testing greenhouse conditions concerning light, temperature etc.; economic optimization.
- Testing the formation and ripening of fruits from the flower to the commercial product by means of residual chlorophyll fluorescence.
- Testing environmental conditions influenced by pollution.
- Testing the behaviour of ecosystems upon global changes (e.g. of CO<sub>2</sub>, O<sub>3</sub>, temperature, volatile organic compounds, UV).

**Requirements for a useful fluorescence test**

Anyone who goes into a forest with good ideas, created in the laboratory in order to analyse a tree, recognizes that it does not take long before many problems, which were not predictable, become apparent. Whatever the tree is, a sample has to be selected first. There is no such problem in the laboratory, as nice, homogeneous material can be grown there. But standing in front of a tree we have to take many subjective decisions concerning which leaf or branch to choose, since every type of leaf material, from green to brown, with or without parasites, may be found on every tree. The choice becomes even more complicated when dealing with an ecosystem.

The answer is that many samples have to be chosen. However, measuring all these many samples takes a lot of time during which the samples may change their behaviour due at least to diurnal changes. Therefore, a fast screening procedure is needed to provide an overall picture of the vitality and the fitness of the samples. Deviation from normality can then be localized so that eventually the time-consuming more specific and accurate investigations can be done. For such a fast screening procedure, a single measurement must take not more than 20 seconds, so that 100 or more samples can be measured per working hour. The instrument has to be lightweight and the batteries have to hold many hours in the field under any weather conditions. It should also be easily handled so that even non-specialists can collect and store the data.

There is not a big choice of instruments and assays fulfilling these requirements. We have chosen the portable fluorometer PEA (Plant Efficiency Analyser, built by Hansatech, King's Lynn, UK) by which the fast Chl *a* fluorescence kinetics can be measured, *in vivo* and *in situ*, with a 10  $\mu$ s time resolution and a measuring time of one second. In this way a new sample can be measured every 10 seconds.

**Reduction and standardization of parameters**

The fluorescence transient is a signal extremely rich in terms of quality and quantity of different information. This complexity bears already a problem concerning the interpretation of the signal, to which the heterogeneity of the biological sample is added. It is therefore necessary to reduce and standardize other parameters, otherwise it would be impossible to interpret the transients from a big scale screening field experiment.

In the following we will describe the physiological state of the sample as dark-adapted (marked as <sup>d</sup>) or light-adapted (marked as <sup>l</sup>). However, for each case the conditions have to be defined and standardized, i.e. the duration of the dark or the light adaptation, as well as the light quality and intensity for the light adaptation. Concerning the latter, the light sources incorporated in the fluorometer must be used to illuminate the sample in the field in a well-defined way, as the actual light conditions vary all the time.

A light-adapted (<sup>l</sup>) or dark adapted (<sup>d</sup>) sample is treated by two types of calibrated light sources:

- (a) Saturating light: The physiological reactions are light saturated. Higher light doses may result in photodamage. In many situations, even under strong light conditions, an adaptation may be achieved and, thus, a steady state may be observed.

- (b) Light-limiting conditions: The sample adapts to the given light intensity, exhibiting a steady state behaviour in the (limited) light-adapted state.

### **The polyphasic fluorescence rise from $F_0$ to $F_M$**

Any photosynthetic sample at any physiological state exhibits upon illumination a fast fluorescence rise from an initial fluorescence intensity  $F_0$  to a maximal intensity  $F_p$ . The latter depends on the intensity of the illumination and becomes highest under saturating light conditions, denoted then as  $F_M$ . Between these two extrema the fluorescence intensity  $F_t$  was found to show intermediate steps like  $I_1$  and  $I_2$  (Neubauer and Schreiber, 1987) or  $F_j$  at about 2 ms and  $F_i$  at about 30 ms (Strasser and Govindjee 1992a,b; Strasser *et al.*, 1995), while  $F_M$  is reached after about 300 ms. There are also cases that the full transient shows a sequence of more steps, e.g.  $F_0$ -J-I-H-G (Tsimilli-Michael *et al.*, 1998). In some heat-stressed samples an additional step K (Guissé *et al.*, 1995; Srivastava *et al.*, 1997; Strasser, 1997) appears at about 300  $\mu$ s leading to a transient  $F_0$ -K-J-I-P. The K-step appears also in plants in their natural habitat (Srivastava *et al.*, 1997). The labeling of the steps follows an alphabetic order, from the slower to the faster part of the transient. Any step could be the highest and would then become  $F_p$ , or even  $F_M$  under saturating light conditions.

### **Physiological state changes**

A physiological state is defined by the molecular composition and the conformation of the sample and it can be described by a constellation of structural and conformational parameters. Every environmental change forces the photosynthetic system to adapt by changing its physiological state (for an extended paper see Tsimilli-Michael *et al.*, 1996). This is also reflected in the shape of the fast polyphasic fluorescence transient which has been shown to change upon changes in various environmental conditions, such as light intensity (Tsimilli-Michael *et al.*, 1995; Srivastava and Strasser, 1996; Krüger *et al.*, 1997), temperature (Guissé, *et al.*, 1995; Srivastava *et al.*, 1997; Strasser, 1997), drought (Van Rensberg *et al.*, 1996) or chemical influences (Ouzounidou *et al.*, 1997).

Adaptation is an expression of a survival strategy and, therefore, the vitality of a photosynthetic system can be investigated by studying its adaptability, for example to changing light conditions.

It is assumed that the fluorescence rise is very fast to permit any change of the physiological state of the sample. This means that the structure and conformation of the sample remain constant and the variation in the fluorescence intensity are solely due to changes in the redox state of the reaction centre complex of PSII. Thus, for a dark-adapted sample all the steps in the transient refer to the same dark-adapted state and can be denoted as  ${}^dF_0$ ,  ${}^dF_j$ ,  ${}^dF_i$ ,  ${}^dF_p$  or  ${}^dF_M$ , or any other  ${}^dF_t$ , and for a light-adapted sample all the steps refer to the same light-adapted physiological state and can be denoted as  ${}^lF_s$ ,  ${}^lF_j$ ,  ${}^lF_i$ ,  ${}^lF_M$  or any other  ${}^lF_t$ . Therefore, by performing the fluorescence tests of a sample when it is dark-adapted and after being light-adapted, the comparison of the two transients and of the corresponding parameters which can be calculated from the raw data allows to estimate the capability of a sample to adapt from dark to certain light conditions. A similar test can be done to follow the adaptation from one light condition to another.

## The experimental fluorescence signal

### *How to measure the fluorescence transient*

Fluorescence spectroscopy is today of high importance for many research fields. Many fluorescence techniques have been developed and a large number of fluorimeters are commercially available. In many laboratories additional home-made instruments with particular performances can be found. However, in this report we will only concentrate on techniques for measuring fluorescence kinetics suitable for a screening test of a large number of samples.

Two techniques remain practical: direct fluorescence and modulated fluorescence. Both techniques have advantages and limitations which, upon the continuous technological development, have favoured the one or the other technique in a somehow periodic manner. For a given state any fluorescence signal is proportional to the excitation light intensity. Therefore, low sensitivity of photocells and photomultipliers required high excitation light intensity. This favoured the use of direct fluorescence techniques (for the principle of direct fluorescence, see any textbook on fluorescence), however, with two limitations: (a) the strong excitation light provokes a very fast fluorescence rise; (b) the initial fluorescence  $F_0$  was mostly not measurable due to the long opening time (about 1 ms) of the light shutter.

The development of sensitive photocells, photodiodes and high performance amplifiers allowed the measurement of fluorescence signals with very low excitation light intensity. A low intensity modulated light beam excites measurable fluorescence. The initial and maximal fluorescence can now be measured with high precision. Still, there are two limitations: (a) the time resolution is limited by the modulation and demodulation frequencies; (b) a low cost modulated fluorimeter with 10  $\mu$ s time resolution and 12 bit signal resolution is not available. Today, shutterless systems with optoelectronic parts make the direct fluorescence measurements very fast with 10  $\mu$ s time resolution and 12 bit signal resolution, as for example the Plant Efficiency Analyser (PEA, by Hansatech; resolution of 1 to 5  $\mu$ s for the commercial version of 1998). It consumes very low power and is of low cost. The high resolution of the fluorescence rise allows one to observe the several steps,  $F_0$ , K, J, I, P, etc. New LEDs (light emitting diodes) allow to select the light quality (even white light). The only limitation is that shielding of ambient light is needed. So, at the present time the direct fluorescence technique has several advantages over the modulated technique:

- much more information per measuring time
- longer independence while working in the field (low power consumption)
- high sample testing rate ( $\sim$  100–300 samples per working hour)
- data collection under any weather conditions (under sea as well)
- light weight
- less than half the cost of any comparable modulated system.

Laser-induced modulated fluorescence imaging is and will remain helpful (like modulated fluorescence kinetics) in tackling many specific questions. The major advantage of modulated fluorescence is the fact that the redox state of the reaction centre complex of PSII can be measured (after a strong light pulse) in the dark (only with a very weak modulated excitation beam).

In the near future, new systems for routine analysis and screening will be available, by which imaging of a dynamic 3D fluorescence will be possible, each pixel of the image behaving like a direct fluorescence signal. Mass storage and data handling will allow remote sensing of fluorescence imaging by satellites.

### The experimental signals $F_0$ to $F_M$

To obtain an experimental signal between  $F_0$  to  $F_M$ , a dark-adapted leaf is illuminated with a saturating light pulse (duration of saturating light pulse: 500 ms to 10 s). The duration of the light pulse has to be chosen in such a way that the maximal fluorescence can be detected ( $F_M$  is reached mostly before 500 ms). The information gathered by the two systems (modulated and direct) are as follows.

#### Modulated fluorescence signal with two strong light pulses

Figure 25.2 shows the modulated fluorescence measurements on a pea (*Pisum sativum*) leaf. The saturating light pulse makes it possible to measure  ${}^dF_0$  and  ${}^dF_M$  of the dark-adapted sample and  ${}^lF_S$  and  ${}^lF_M$  of the light-adapted sample (the real  $F_0$  fluorescence for the light-adapted state can only be measured by using an additional far-red light source). Often  ${}^lF_0$  is estimated to be close to  ${}^dF_0$ . Only a few expressions need this value and for routine measurements such an approximation is acceptable. Between the two saturating light pulses the light-adaptation transient under light-limiting conditions is shown in Figure 25.2. In a routine test the four values  ${}^dF_0$ ,  ${}^dF_M$ ,  ${}^lF_S$  and  ${}^lF_M$  can be easily measured

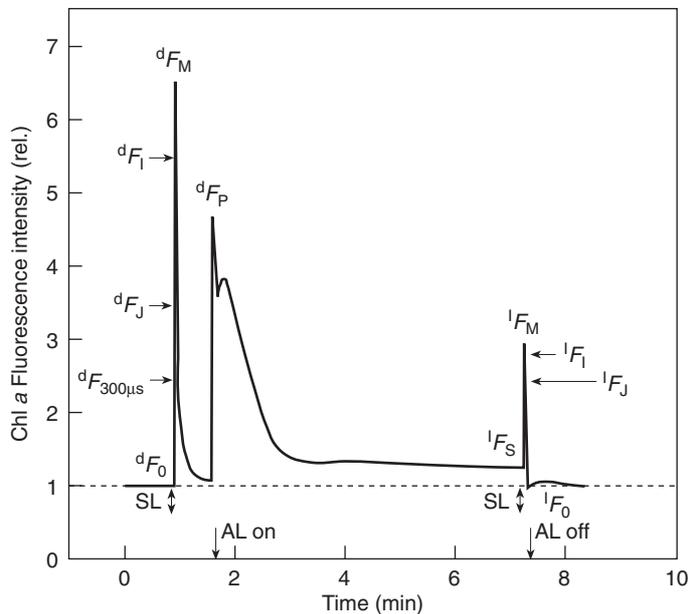


Figure 25.2 An example of a modulated fluorescence measurement. The selected fluorescence values on the fast rise are taken from the direct fluorescence measurement of the same sample (transient of Figure 25.1). SL, saturating light; AL, actinic light.

by the 1s pulses with a modulated fluorometer (Table 25.2, shaded field only). The light state, however, depends on the light intensity used during the light adaptation (Havaux *et al.*, 1991).

*Direct fluorescence signals with two strong light pulses*

Parallel measurements with those of Figure 25.2 were carried out by the fast direct fluorometer PEA (Figure 25.3). The time resolution is 10 μs with the first reliable data point at 40 μs. The transient from  $F_0$  to  $F_p$  or  $F_M$ , which appears only as a spike in the currently used modulated techniques, reveals now clearly the steps J and I when presented on a logarithmic time scale. Moreover, very accurate fluorescence slopes can be measured at any time.

Table 25.2 Fluorescence data from a routine test carried out with modulated fluorescence measurements (shaded field) or direct fluorescence measurements (white and shaded field) using two strong light pulses, one before and one after 10 min of light adaptation.

Dark adapted	$dF_0$	$dF_{300\mu s}$	$dF_J$	$dF_I$	$dF_P$	$dF_M$	$dArea$	$dt_{Fmax}$	$dF_0/dF_M$
	I	2.56	3.56	5.43	4.72 *	6.52	97.0	203 ms	0.15
Light adapted	$I F_0^{**}$	$I F_{300\mu s}$	$I F_J$	$I F_I$	$I F_S$	$I F_M$	$I Area$	$I t_{Fmax}$	$I F_0/I F_M$
	—	1.96	2.33	2.80	1.62	2.92	22.2	873 ms	0.35

\* See second strong light pulse in Figure 25.2.

\*\* A far-red light source is needed.

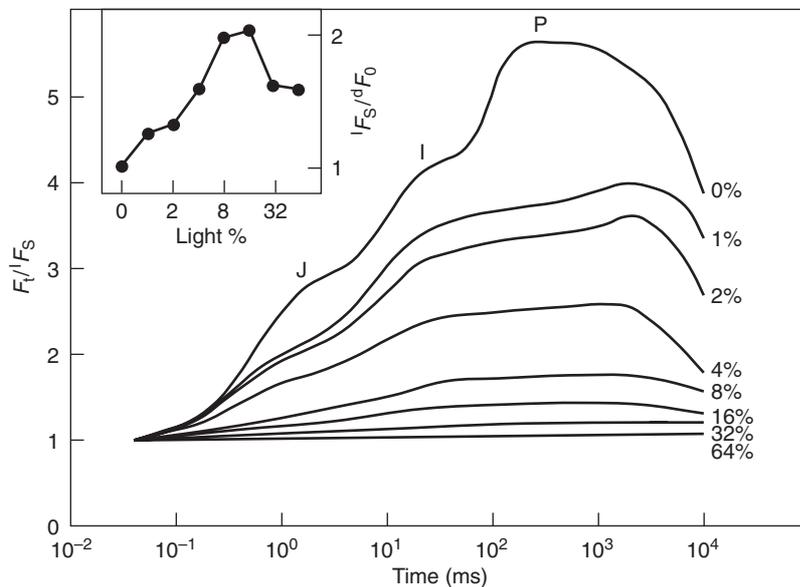


Figure 25.3 Examples of direct fluorescence measurements with a PEA instrument. The fluorescence transients (measured with 100% light), plotted on a logarithmic time scale, were measured on pea leaves at different light adapted states (0% to 64% of 600 Wm<sup>-2</sup> for 10 sec).

In Figure 25.3, beside the fluorescence transient of the dark-adapted sample (indicated by 0%), the transients after light adaptation are also presented (see also Srivastava *et al.*, 1995). The actinic light used for the light adaptation was provided by the light source of the PEA instrument (red light with peak at 650 nm, of maximum  $600 \text{ Wm}^{-2}$  intensity = 100%). The different intensities of actinic light used for light adaptation, expressed as percentages of the maximum intensity provided by the source, are shown for each transient. The easily obtainable data of the fluorescence rise during a light pulse are shown in Table 25.2 (white and shaded areas).  $F_0$  is measured at 50  $\mu\text{s}$ . Therefore, the slope at the origin can be calculated as a fluorescence increment per ms, e.g., from 50  $\mu\text{s}$  to 300  $\mu\text{s}$  as  $(\Delta F/\Delta t)_0 = 4 * (F_{300\mu\text{s}} - F_{50\mu\text{s}})$ .

Several expressions utilizing such a selection of data have been derived, and are described below. These expressions are shown in Table 25.3 which also presents, as an example, their values calculated from the data of Table 25.2. The expressions and values utilizing the raw data from the modulated fluorescence measurement are indicated by shaded areas while the whole of the expressions and values of Table 25.3 (white and shaded areas) can be calculated from a direct, as above presented, fluorescence measurement.

#### **Direct fluorescence signals with multiple strong light pulses**

The full fluorescence transient under a given actinic light intensity (light-adaptation transient) is usually measured by modulated fluorescence techniques. Repetitive high light pulses of 0.5 to 1 s duration are applied to detect for each light pulse the maximal fluorescence intensity  $F_M$  of the sample, i.e. the fluorescence intensity when all the reaction centers are closed.

Fast fluorescence techniques (light measurements with the PEA instrument) allow one to measure this information as well. Moreover, they provide in digital form the fluorescence kinetics of the full closure of the reaction centers, i.e., the kinetics from the actual fluorescence intensity under actinic light up to the fluorescence intensity  $F_M$  (e.g.,  $F_0$ ,  $F_J$ ,  $F_I$ ,  $F_M$ ). However if a strong light pulse is given to a sample already excited with actinic light the initial fluorescence signal  $F_0$  becomes a measure for the maximum  $F_p$  or steady state fluorescence signal,  $F_s$ . The same is valid for any state between  ${}^4F_p$  and  ${}^4F_s$  (see Figure 25.4). Such an example of a full light-adaptation transient is shown in Figure 25.4. The adaptation time was 10 min and the actinic light intensity was set at 3% of the maximal intensity,  $600 \text{ Wm}^{-2}$ , provided by the light source of the PEA instrument. Every 10 sec a light pulse of 1 sec duration with an intensity of 100% was given. Each such pulse created a fast closure of all reaction centers, reflected in a fast fluorescence transient which was recorded as more than 1000 digitized points with the PEA instrument. The insert of Figure 25.4 shows (A) the first (dark-adapted) and (B) the last (10 min light-adapted) fast fluorescence transient. Each vertical trace in Figure 25.4 corresponds to such a transient  $F_0$  (or  $F_s$ )  $F_J$ ,  $F_I$ ,  $F_M$ . For clarity only every 5th data point is plotted in these transients.

From Figure 25.4 the following raw data are selected:

$$\begin{aligned} \text{Curve A:} & \quad {}^4F_0 = 0.453 \quad {}^4F_V = 2.975 \quad {}^4F_M = 3.428 \quad {}^1F_0 = 0.453 \\ \text{Curve B:} & \quad {}^1F_s = 0.85 \quad {}^1F_V = 1.081 \quad {}^1F_M = 1.534 \end{aligned}$$

Table 25.3 Calculated expressions using the data of Table 25.2, from modulated fluorescence measurements (shaded field) or direct fluorescence measurements (white and shaded field).

Dark adapted	${}^dS_m$	${}^dV_J$	${}^dV_I$	$(d{}^dV/dt)_0$	${}^d\phi_{Po}$	${}^dABS/RC$	${}^dTR_0/RC$	${}^dET_0/RC$	$N$
	17.57	0.46	0.80	0.28	0.85	2.87	2.43	1.30	10.7
Light adapted	${}^lS_s^*$	${}^lV_J$	${}^lV_I$	${}^lV_s$	${}^l\phi_{Po}$	${}^l\phi_{Ps}$	${}^lq_N$	${}^lq_P$	${}^lS_s/t_{Fmax}$
	11.55	0.69	0.94	0.32	0.66	0.45	0.65	0.68	0.013

\* The index "S" in  ${}^lS_s$  refers to the light-adapted stationary state and not to the single turnover situation defined in the section "The area growth above the fast fluorescence rise".

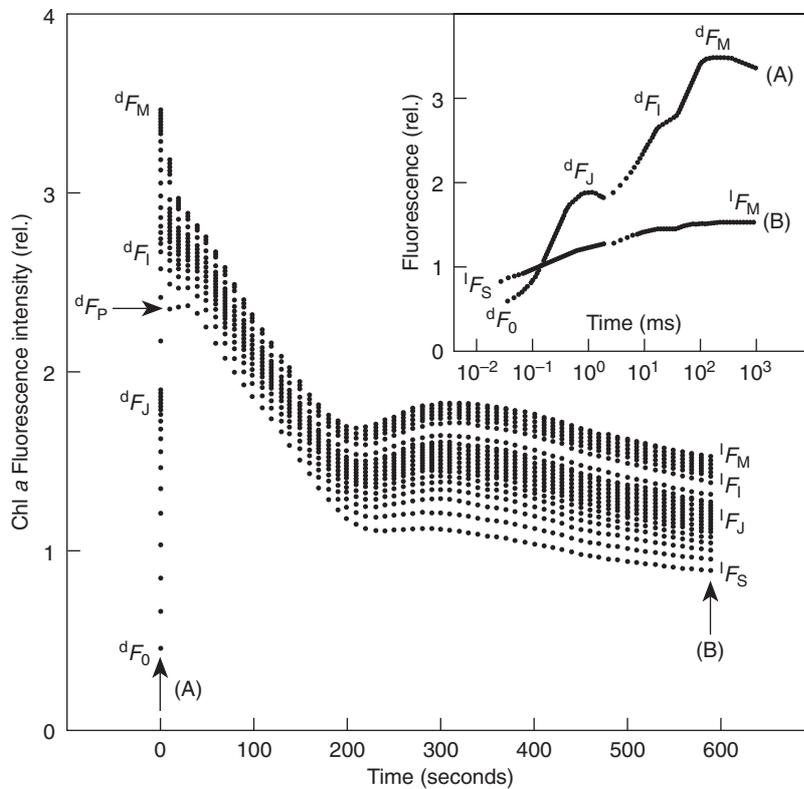


Figure 25.4 The light-adaptation transient of a dark-adapted pea leaf. The adaptation time was 10 min and the actinic light intensity was set at 3% of the maximal intensity,  $600 \text{ Wm}^{-2}$ , provided by the light source of the PEA instrument. Every 10 s during the actinic illumination, a light pulse of 1 s with an intensity of 100% was applied which provoked a fast fluorescence rise, measured and digitized between  $10 \mu\text{s}$  to 1 s by the PEA instrument. The inset shows the fast fluorescence rise of the first (A) and the last (B) light pulses. Each vertical trace in the main figure corresponds to a complete fast fluorescence rise. The two envelop lines, of all the highest and all the lowest fluorescence values, correspond to the fluorescence transients under actinic light of  $F_M$  and  $F_P$  respectively. The latter reaches finally a stationary value labeled  ${}^lF_s$ . All intermediary steps  $F_J$  or  $F_I$  are available from the data files.

Based on these data several expressions can be calculated which describe the behaviour of the sample (Table 25.4). These expressions will be discussed on the following pages. However, it is worth pointing out here that the most fashionable expressions  $q_N$  and  $q_P$  (the so-called nonphotochemical and photochemical quenching, respectively) can be easily obtained by direct fast fluorescence techniques as shown in Table 25.4, and not only from modulated fluorescence measurements, as mostly reported.

## Processing fluorescence data

### Empirical processing of data

#### Only the extrema $F_0$ and $F_M$ are used

The fast fluorescence rise starts at the initial low value  $F_0$  and reaches a maximal value  $F_M$ . From these two values, several expressions can be calculated. The basis of all these expressions is the ratio  $F_0/F_M$  and the difference  $F_V = F_M - F_0$  of the two values. Figure 25.5 shows how all formulations combining the fluorescence signals of  $F_0$  and  $F_M$  can be converted into one another. This equivalency points out that the several expressions cannot be used independently and, further, cannot lead to different interpretations when fluorescence data are discussed. Each of these expressions is a combination of the two signals  $F_0$  and  $F_M$ . Therefore, all these combinations carry one identical piece of information. The only difference is in the scaling of the values.

#### Full kinetics of $F_t$ from $F_0$ to $F_M$

The full kinetics of the fluorescence rise is given by the fluorescence values  $F_t$  at any time  $t$ . Different samples may exhibit fluorescence signals of different amplitude.

Table 25.4 Expressions describing the behaviour of a sample and their calculated values from the data of Figure 25.4, obtained by direct fast fluorescence techniques.

Biophysical meanings	Correlation of symbols and fluorescencesignals	Empirical definition	Values from figure	Data of curves
Maximum yields of primary photochemistry (dark-adapted)	${}^d\varphi_{Po} = {}^d k_p / ({}^d k_p + {}^d k_N) = 1 - {}^d F_0 / {}^d F_M$		0.87	A
Maximum yield of primary photochemistry (light-adapted)	${}^l\varphi_{Po} = {}^l k_p / ({}^l k_p + {}^l k_N) = 1 - {}^l F_0 / {}^l F_M$		0.70	B
Actual yield of primary photochemistry (light-adapted)	${}^l\varphi_{Ps} = {}^l\varphi_{Po} (1 - {}^l V_s) = 1 - {}^l F_s / {}^l F_M$		0.45	B
Relative ratio of photochemical and non-photochemical rate constants	$\frac{{}^l k_p / {}^l k_N}{{}^d k_p / {}^d k_N} = \frac{{}^l\varphi_{Po} / (1 - {}^l\varphi_{Po})}{{}^d\varphi_{Po} / (1 - {}^d\varphi_{Po})} = \frac{{}^l F_V / {}^l F_0}{{}^d F_V / {}^d F_0}$	$1 - q_N$	0.36	A, B
Relative variable fluorescence (light-adapted) *	$V_s = \frac{B}{1 + p_G (k_p / k_N) (1 - B)} = \frac{{}^l F_s - {}^l F_0}{{}^l F_M - {}^l F_0}$	$1 - q_P$	0.36	B

Here,  $k_p$  and  $k_N$  refer to the de-excitation rate constants of photochemical and non-photochemical events. In the formula expressing  ${}^l V_s$ ,  $B$  is the fraction of closed reaction centers and  $p_G$  is the overall probability for the energetic cooperativity between PSII antenna of different photosynthetic units (grouping).

\* Replacing  ${}^l F_0$  by  ${}^d F_0$  and  ${}^l F_M$  by  ${}^d F_M$  and  ${}^d F_s$  by  $F_p$  of the second light pulse allows to calculate the highest relative variable fluorescence ( ${}^d V_p$ ) which can be reached by the actinic light intensity used for a dark-adapted sample. For definition of other symbols, see the next section.

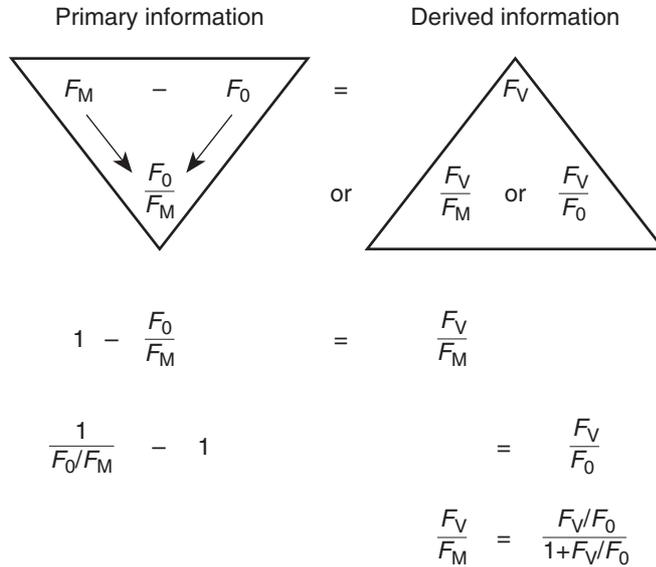


Figure 25.5 A demonstration of how the several expressions combining the extrema  $F_0$  and  $F_M$  are converted into one another.

However, with proper normalization it is possible to bring these signals into such a form that they can be compared with one another. In Figure 25.6 a combination of normalization methods is shown. New symbols can be used to substitute fluorescence expressions:

- $\varphi_{p_0}$  substitutes expressions containing only  $F_0$  and  $F_M$
- $\varphi_{p_t}$  substitutes expressions containing only  $F_t$  and  $F_M$
- $V_t$  substitutes expressions containing  $F_0$ ,  $F_t$  and  $F_M$ .

*Empirical equation of any fluorescence rise*

It is a challenge to describe the fluorescence rise in an empirical way, i.e., without knowing what the components of an expression mean in terms of physics and biology. Even so, a phenomenological quantification of the fluorescence behaviour of a sample can be made and used to compare and classify different samples. Such an empirical expression for any signal  $F_t$  or any normalization of it, like  $F_t/F_M$  or  $F_t/F_0$ , is presented in Figure 25.7.

It appears that all the information needed to describe the shape of the variable part of a fluorescence kinetics is given by the kinetics of the relative variable fluorescence, defined as  $V_t = (F_t - F_0)/(F_M - F_0)$ . The variable part of any fluorescence induction kinetics can thus be presented on a scale from zero to unity ( $0 \leq V_t \leq 1$ ). The biophysical understanding of this function,  $V_t$ , is therefore at the centre of interest. (For more details, see energy flux theory in biomembranes by Strasser, 1978.)

Any fluorescence value in time	Only extrema of fluorescence	Complement to unity of relative variable fluorescence
$\frac{F_M - F_t}{F_M}$	$\frac{F_M - F_0}{F_M}$	$\frac{F_M - F_t}{F_M - F_0}$
$1 - \frac{F_t}{F_M}$	$1 - \frac{F_0}{F_M}$	$1 - \frac{F_t - F_0}{F_M - F_0}$
$\phi_{Pt}$	$\phi_{Po}$	$1 - V_t$

Figure 25.6 Expressions normalizing the full kinetics of  $F_t$  from  $F_0$  to  $F_M$ , where the relative variable fluorescence  $V_t = (F_t - F_0) / (F_M - F_0)$ .

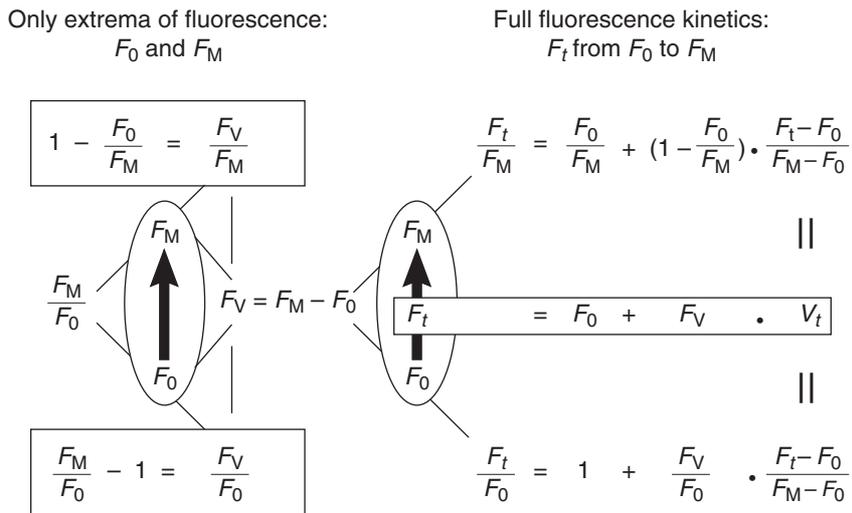


Figure 25.7 The derivation of the relative variable fluorescence  $V_t$  for an empirical quantification of the fluorescence signal. Only  $V_t$  determines the dynamic shape of any fluorescence transient.

As in any field of science, the experimental signals can be processed in a purely phenomenological way, so that practical expressions can be derived and defined. Figure 25.8 shows such phenomenological signal processing. Two phenomenological presentations are given. First the experimental signals are directly used and second the fluorescence signals  $F_t$  are converted into  $\phi_{Pt}$  signals according to the substitution  $\phi_{Pt} = (F_M - F_t) / F_M$ . It is thus possible to draw all fluorescence signals, measured at any time, as a function of  $\phi_{Pt}$  versus  $V_t$ . Both parameters,  $\phi_{Pt}$  and  $V_t$ , can take values between 0 and unity only.

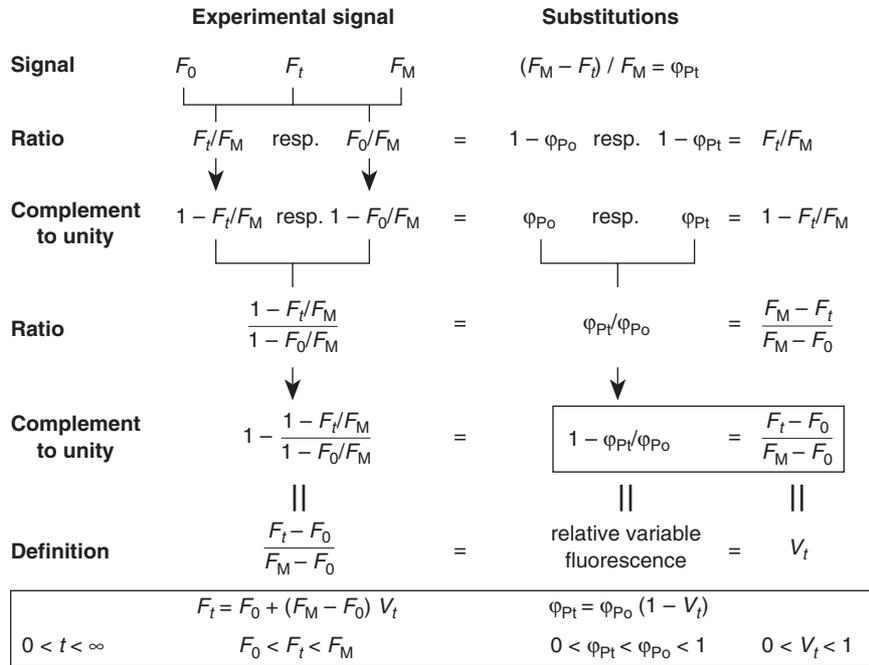


Figure 25.8 A phenomenological signal processing by which the experimental signals  $F_t$  are either directly used or converted into  $\varphi_{Pt}$  or  $V_t$  signals.

*The empirical behaviour of a plant during light-adaptation*

At each physiological state a sample is characterized generally by  $F_0$ ,  $F_t$  and  $F_M$ . However, in Figure 25.1 we have seen that the full fluorescence rise exhibits particular steps like  $F_J$  (at about 2 ms) and  $F_I$  (at about 30 ms). In the light we have, in addition, the stationary state  $F_S$ .

Therefore, the infinite points of a fast fluorescence rise can be reduced to the following fluorescence signals for each physiological state:

dark-adapted state:	${}^dF_0$ ${}^dF_J$ ${}^dF_I$ ${}^dF_M$
light-adapted state:	${}^lF_S$ ${}^lF_J$ ${}^lF_I$ ${}^lF_M$

From the normalized expressions,

- of the extrema:  $\varphi_{Po} = 1 - F_0/F_M$  and
  - the relative variable fluorescence:  $V_J = (F_J - F_0) / (F_M - F_0)$   
 $V_I = (F_I - F_0) / (F_M - F_0)$   
 $V_S = (F_S - F_0) / (F_M - F_0)$
- and applying the relation  $\varphi_{Px} = \varphi_{Po} \cdot (1 - V_x)$   
 we get:  $\varphi_{PJ} = \varphi_{Po} \cdot (1 - V_J)$   
 $\varphi_{PI} = \varphi_{Po} \cdot (1 - V_I)$   
 $\varphi_{PS} = \varphi_{Po} \cdot (1 - V_S)$

all belonging to the same physiological state (Havaux *et al.*, 1991).

We show here, as an example, how these expressions can be used to describe the phenomenological behaviour of a sample upon light adaptation. A dark-adapted sample was exposed and adapted to different light intensities (actinic light intensities). At every adapted physiological state the fluorescence transient was recorded and all of the above expressions were calculated from the selected fluorescence signals. A first observation is that the ratio  $F_0/F_M$  is higher in the light-adapted states and that, the higher the actinic light intensity the more pronounced this increase is. Theoretically the ratio  $F_0/F_M$  can take any value between 0 and 1. Therefore, the ratio  $F_0/F_M$  can be used as an indicator of the physiological state (state change index) which is thus calibrated on an axis between 0 and 1.

Figure 25.9 shows these empirical expressions, for each light-adapted state, as a function of the light intensity of adaptation (actinic light intensity) and also as a function of the state index  $F_0/F_M$ . The light intensity is expressed as a percentage of the highest light intensity used ( $600 \text{ Wm}^{-2}$  of red light of  $650 \pm 30 \text{ nm}$ ).

Any expression describing one physiological state can be compared with the corresponding expression referring to another physiological state. The dark-adapted state can well serve as a reference state for the normalization of the light-adapted states. An example is the expression

$$q_N = 1 - [(F_V/F_0)_{\text{light}} / (F_V/F_0)_{\text{dark}}]$$

which is often used in the literature.

All expressions thus far, have been derived in a purely empirical way, i.e. no theory at all has been applied and up to now no biological or biophysical meaning has been given to any of them. However, all these expressions can be used to present and compare a biological sample in many types of experiments.

### **Conceptual processing of data**

In order to link the experimental signals to biological reaction mechanisms, one has to choose and apply a theory. Any theory, however, is based on certain concepts in terms of physics and chemistry and their link to the behaviour of a sample. The theory chosen may describe the sample in a general or in a well-defined way. The investigator has to decide which level of complexity is appropriate for the given situation.

#### *The actual dogmatics of the fluorescence emission of PSII*

The knowledge in this field is very advanced. In this chapter, however, we just focus on the concepts which help to understand the *in vivo* fluorescence transient of plants. As a simplification we consider the following components of PSII:

- all absorbing pigments (mainly chlorophylls, Chl)
- the reaction centre, RC
- the primary quinone electron acceptor,  $Q_A$
- all the electron carriers beyond  $Q_A$ .

The photon flux absorbed by the pigments is indicated as ABS; the dissipated flux (all

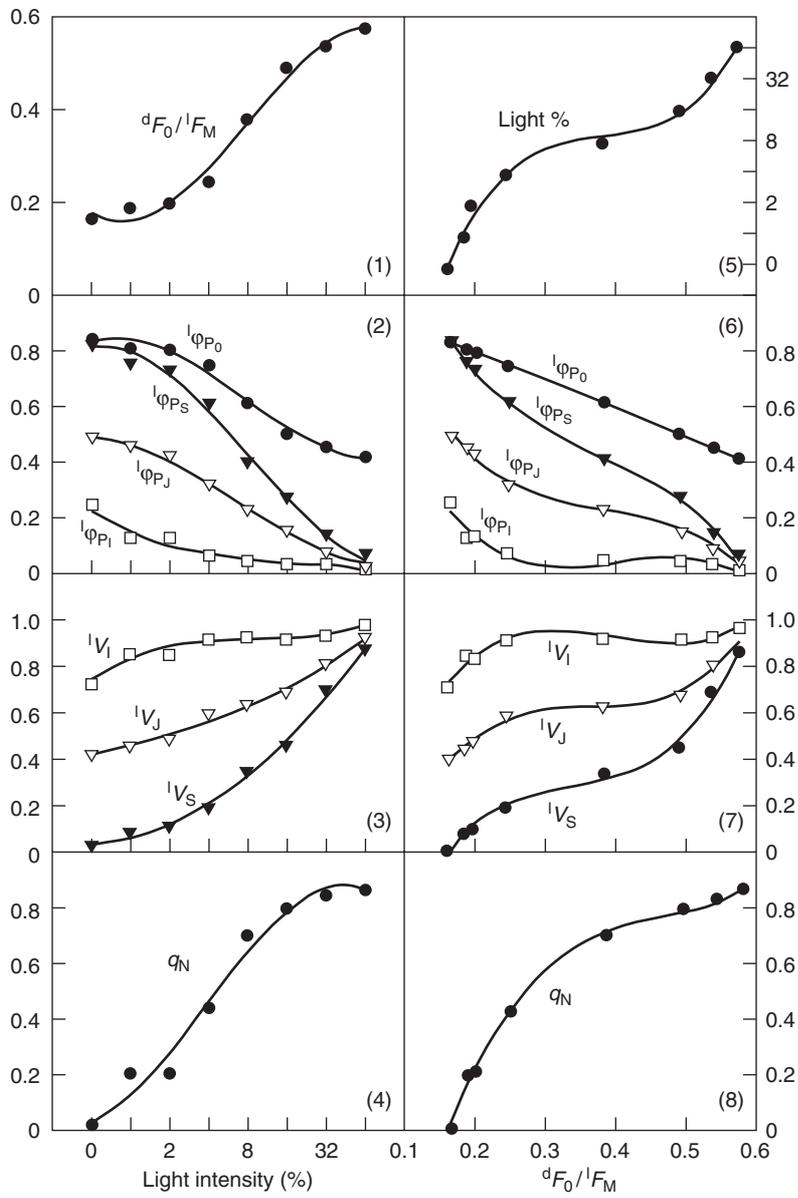


Figure 25.9 An example of how the several empirical expressions, presented vs the actinic light intensity and vs the state index  $dF_0/I_{FM}$ , can describe the phenomenological behaviour of a sample upon light adaptation. All the curves shown here can be obtained with direct fluorescence technique (PEA, Hansatech). Usually, modulated techniques allow only measurements of curves presented with closed symbols.

the de-excitations except the photochemical one) is indicated as DI, which includes as well the fluorescence emission, indicated as F; the excitation energy flux which reaches the RC and gets trapped there (in the sense of leading to  $Q_A$  reduction) is indicated as trapping flux TR; the energy flux corresponding to the electron transport beyond  $Q_A^-$  is indicated as ET.

When  $Q_A$  is in its oxidized form the excited reaction centre is called open ( $RC^{op}$ ), as it can promote the reduction of  $Q_A$  to  $Q_A^-$ , thus converting excitation energy into free energy of the redox couple  $Q_A^-/Q_A$ . If  $Q_A$  is already reduced, then the excited reaction centre is called closed ( $RC^{cl}$ ). Therefore, a PSII unit with open RC can be indicated as Chl.RC. $Q_A$  and a PSII unit with closed RC as Chl.RC. $Q_A^-$ .

Dogmatically, it is accepted that, for the same physiological state (i.e. dark-adapted or light-adapted):

- an excited PSII with an open RC shows a low fluorescence emission;
- an excited PSII with a closed RC shows a high fluorescence emission.

As a working hypothesis it has been generally considered that a sample in darkness has only open RCs and that a sample in strong light has only closed RCs. Therefore, the fluorescence rise from  $F_0$  to  $F_M$  corresponds to the reduction of  $Q_A$  to  $Q_A^-$ , starting with all RCs open until all the RCs are closed. One has to keep in mind that the redox state of plastoquinone (PQ or  $PQH_2$ ) can influence the fluorescence intensity mainly at  $F_M$ . For simplicity, the possible quenching by PQ is not considered in the following equations.

#### Correlation of fluorescence signals with energy fluxes

The total light energy flux absorbed by a sample can be split into two fractions:

- the energy flux which is conserved as free energy by the primary photochemical reaction, denoted as trapping flux TR; and
- the energy flux which is dissipated, DI, as heat and fluorescence, or transferred to other systems.

We can, therefore, write:

$$ABS = TR_t + DI_t \quad (25.1)$$

For samples which, previous to their excitation, had all reaction centers open (e.g., in the dark state) we can make the following link to the fluorescence rise:

- at  $t = 0$ ,  $F \equiv F_0$  is minimal and  $TR \equiv TR_0$  is maximal;
- at  $t = \infty$  (practically at  $t = t_{Fmax}$ ),  $F \equiv F_M$  is maximal and  $TR \equiv TR_M = 0$ .

Assuming that the fluorescence emission is proportional to the total dissipation,  $F_t \sim DI_t$ , and introducing a proportionality factor  $\alpha$ , we can write:  $DI_t = \alpha F_t$ . Therefore,

$$ABS = TR_t + \alpha F_t \Rightarrow F_t = (ABS - TR_t) / \alpha \Rightarrow F_M = ABS / \alpha \quad (25.2)$$

Hence, the normalized signal  $F_t/F_M$  is linked with the flux ratio  $TR_t/ABS$ :

$$F_t/F_M = 1 - (TR_t/ABS) \quad (25.3)$$

However, the flux ratio  $TR_t/ABS$  expresses the ratio of an energy output from the antenna pigments to the photon input into the antenna pigments and, therefore, corresponds to the yield of excitation energy trapping  $\varphi_{pt}$ , known also as the yield of primary photochemistry or the photon use efficiency. Hence,

$$\varphi_{pt} = TR_t/ABS = 1 - F_t/F_M \quad (25.4)$$

(Note that  $\varphi_{pt}$  is not any more an empirical expression but a biophysical one).

The maximum yield of primary photochemistry,  $\varphi_{po}$ , is therefore written as:

$$\varphi_{po} = TR_0/ABS = 1 - F_0/F_M \quad (25.5)$$

Dividing equation (25.4) by equation (25.5) we get:

$$\varphi_{pt}/\varphi_{po} = (F_M - F_t)/(F_M - F_0) = 1 - [(F_t - F_0)/(F_M - F_0)] = 1 - V_t \quad (25.6)$$

where  $V_t$  is the relative (or normalized) variable fluorescence.

For the general case and at any time we can therefore write for  $\varphi_{pt}$  the following equation:

$$\varphi_{pt} = \varphi_{po} (1 - V_t) \quad (25.7)$$

The term  $\varphi_{po}$  contains only the fluorescence extrema  $F_0$  and  $F_M$  and the term  $\varphi_{pt}$  contains the fluorescence expressions  $F_M$  and  $F_t$ . Therefore,  $V_t$  contains all the three fluorescence signals  $F_0$ ,  $F_t$  and  $F_M$ .

Figure 25.10 shows the historical sequence of appearance in the literature of the equations 25.4 to 25.7 which all link experimental fluorescence signals with the quantum yield of primary photochemistry  $\varphi_p$ .

For the initial conditions of the fluorescence transient (light on and  $t \cong 0$ ) the maximal yield for primary photochemistry expressed as exciton trapped that triggers the reduction of one  $Q_A$  to  $Q_A^-$  per one photon absorbed, can be expressed entirely by using the ratio of the extrema  $F_0$  and  $F_M$ , as shown by equation (25.5). Note that from this equation, several expressions combining the extrema  $F_0$  and  $F_M$  in a different way can be derived, all containing the term  $\varphi_{po}$ . Such expressions are the following, all containing the same information:

$$1 - (F_0/F_M) = \varphi_{po}$$

$$F_0/F_M = 1 - \varphi_{po}$$

$$F_V/F_0 = \varphi_{po} / (1 - \varphi_{po}) = (F_M/F_0) - 1$$

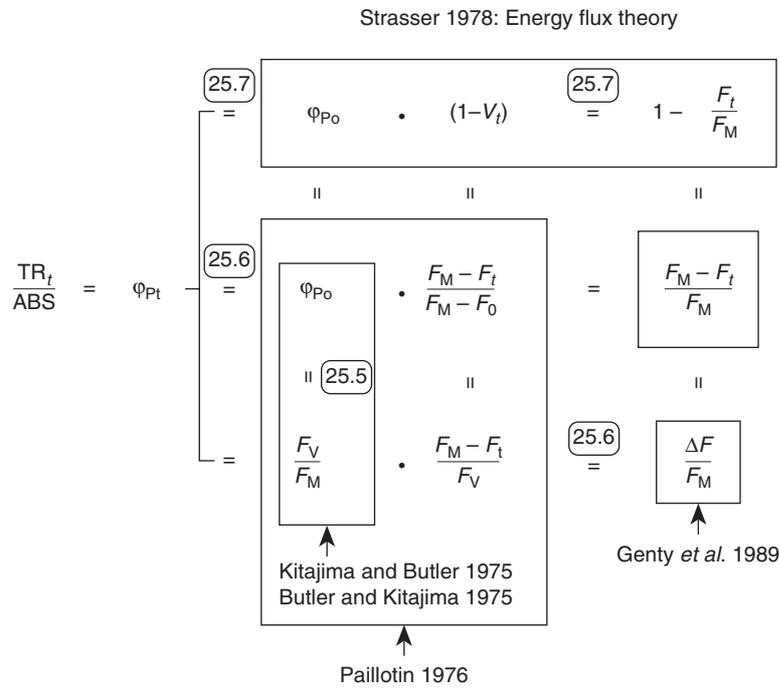


Figure 25.10 The historical sequence of the appearance in the literature of equations (25.4) to (25.7) which all link experimental fluorescence signals to the quantum yield of primary photochemistry  $\phi_p$ .

The above analysis is valid for any physiological state. Among the different physiological states we are here interested mainly in the dark-adapted state (index <sup>d</sup>, e.g., <sup>d</sup>F, <sup>d</sup> $\phi_p$  etc.) and in the light-adapted state (index <sup>l</sup>, e.g., <sup>l</sup>F, <sup>l</sup> $\phi_p$  etc).

*The correlation of a fluorescence ratio and a ratio of rate constants*

Each energy flux, TR, or DI or F, is proportional to the concentration of the excited antenna chlorophyll Chl\* and to the corresponding de-excitation rate constant:

$$\text{flux}_i = \text{Chl}^* \cdot k_i$$

Denoting the sum of all non-photochemical rate constants as  $k_N$  and the photochemical rate constant as  $k_p$ , the sum of all rate constants is written as:

$$\Sigma k_i = k_p + k_N \tag{25.8}$$

Therefore, the absorption flux ABS, being equal to the sum of all the de-excitation fluxes  $k_i \cdot \text{Chl}^*$ , can be written as:

$$\text{ABS} = (k_N + k_p) \cdot [\text{Chl}^*] \quad (25.9)$$

while the maximal trapping flux  $\text{TR}_0$  (when all reaction centers are open) is:

$$\text{TR}_0 = k_p [\text{Chl}^*] \quad (25.10)$$

Hence, equation (25.5) can be now linked to a ratio of the rate constants, as:

$$\text{TR}_0/\text{ABS} = \varphi_{p_0} = k_p / (k_N + k_p) = 1 - (F_0/F_M) = F_V/F_M \quad (25.11)$$

#### *Different ways to express the same thing*

Under *in vivo* conditions it is usually very difficult to measure the concentration of a chemical compound in a photosynthetic sample. However it is possible to record the dynamic behaviour of the sample by its activities and energy fluxes. Thereafter, the ratio of energy fluxes can be correlated with the ratio of fluorescence signals, the quantum yields and the ratio of de-excitation rate constants. Every physiological state can be described in this way. Any expression describing any physiological state can be compared with the corresponding expression referring to another physiological state. Such a comparison can be quantified by the ratio of the two values which thus serves as an index of the state change capability, i.e. of the capability for change from one physiological state to another. This dynamics can be expressed identically in terms of ratios of fluorescence signals, rate constants, yields or energy fluxes, as shown in Figure 25.11 for the change from a dark-adapted to a light-adapted state.

In Figure 25.11 a definition of the expression  $q_N$  is shown as:

$$(F_V/F_0)_{\text{light}} / (F_V/F_0)_{\text{dark}} = 1 - q_N$$

where  $F_V = F_M - F_0$ .

In many situations  ${}^1F_0$  ( $F_0$  in the light) is considered to be close to  ${}^4F_0$  ( $F_0$  in the dark). Therefore,  $q_N$  can be approximated as:

$$“q_N” = 1 - {}^1F_V / {}^4F_V$$

The biological and biophysical meaning of the expression  $q_N$  is shown in Figure 25.11 in terms of ratio of fluxes, ratio of yields, ratio of rate constants and ratio of fluorescence signals. Note that  $q_N$  was mentioned in the section “The empirical behaviour of a plant during light adaptation”, however as a purely phenomenological expression.

#### *The meaning of $q_p$ and $q_N$*

The expression  $q_N$  is usually called in the literature “non-photochemical quenching” to indicate that it quantifies a decrease in fluorescence of an origin different from that of the photochemical quenching  $q_p = (F_M - F) / (F_M - F_0)$ . However, the naming and symbolization lead to confusion, as they give the impression that the two terms are complementary and, moreover, that they refer to the same state. In addition, the characterization “non-photochemical” as such is quite misleading, since  $q_N$  contains as

Physiological state e.g.	Ratio of fluxes	Ratio of yields	Ratio of rate constants	Ratio of fluorescence signals
Dark adapted ↓	$\frac{dTR_0}{dDI_0}$	$= \frac{d\phi_{P_0}}{1 - d\phi_{P_0}}$	$= \frac{d k_P}{d k_N}$	$= \frac{d F_V}{d F_0}$
Light adapted	$\frac{lTR_0}{lDI_0}$	$= \frac{l\phi_{P_0}}{1 - l\phi_{P_0}}$	$= \frac{l k_P}{l k_N}$	$= \frac{l F_V}{l F_0}$
Normalization on a reference state, e.g. on the dark state	$\frac{l(TR_0/DI_0)}{d(TR_0/DI_0)}$	$= \frac{l[\phi_{P_0}/(1-\phi_{P_0})]}{d[\phi_{P_0}/(1-\phi_{P_0})]}$	$= \frac{l(k_P/k_N)}{d(k_P/k_N)}$	$= \frac{l(F_V/F_0)}{d(F_V/F_0)} = \boxed{1 - q_N}$

Figure 25.11 The derivation of the expression  $1 - q_N$ , expressed identically in terms of ratios of fluorescence signals, rate constants, yields or energy fluxes. As an example, it is shown here for the change from a dark-adapted to a light-adapted state.

much photochemical information (through  $k_p$ ) as non-photochemical information (through  $k_N$ ) and, therefore, is not at all a specific index for non-photochemical events (for more details, see Havaux *et al.*, 1991). More arguments can be stated and summarized as follows.

Concerning  $q_p$ :

- $q_p$  is directly defined by the relative variable fluorescence as:  $1 - q_p = V = (F - F_0)/(F_M - F_0)$ ;
- $q_p$  refers to only *one* physiological state;
- $q_p$  depends on the redox state  $Q_A^-/Q_A$  of the sample in a given physiological state; and
- $q_p$  refers by name to the well-defined physical events of photochemical quenching. In other words, the name “photochemical quenching” corresponds precisely to a conceptual interpretation. Even so, it must be pointed out that, when a cooperativity between the PSUs is considered,  $q_p$  depends on the non-photochemical rate constant  $k_N$  as much as on the photochemical one  $k_p$ . This dependence arises from the relation  $V = B / [1 + K(1-B)]$  between the relative variable fluorescence  $V$  and the fraction of closed reaction centers  $B$ , where  $K$  is a constant containing, beside the probability  $p_G$  for cooperativity (grouping), both  $k_p$  and  $k_N$ . If no cooperativity is assumed, the relative variable fluorescence  $V$  is identical to the fraction of closed RC indicated as  $B$  and therefore  $V = B = 1 - q_p$ . However, the rate constant for photochemical quenching  $k_p$  does not change at all, while  $q_p$  can take all values between zero and unity.
- $q_p$  indicates a relative concentration, i.e. the fraction of the open reaction centre  $(1-B)$  if no grouping ( $p_G = 0$ ) is considered.

Concerning  $q_N$ :

- $q_N$  refers to *two* physiological states; it is an index of the change from the dark-adapted to a light-adapted state;
- $q_N$  does not refer to any intermediate redox state  $Q_A^-/Q_A$  of the sample; it is defined only by the fluorescence signals when the reaction centers are all open ( $F_0$ ) and all closed ( $F_M$ ), as in the case of the maximum quantum yield of primary photochemistry,  $\phi_{p_0}$ ;
- $q_N$  depends on  $k_N$  as much as on  $k_p$  (see Table 25.4); and
- $q_N$  is not a specific expression for non-photochemical events. However, in terms of rate constants, changes concerning non-photochemical events can be described by the ratio  ${}^4k_N/{}^1k_N = {}^1F_M/{}^4F_M$  and compared with photochemical events by the ratio  $k_p/k_N = F_V/F_0$ .

#### *Misleading advertisement of $q_p$ and $q_N$*

$q_p$  and  $q_N$  can be used like any useful expression. However care should be taken if interpretations are made. The intensity of the fluorescence from a PSII is higher if the RC does not contribute to photochemistry (hence the definition: closed) than if it does (hence the definition: open). For any fluorescence signal between  $F_0$  and  $F_M$ , one can calculate the relative variable fluorescence  $V = (F - F_0)/(F_M - F_0)$ , which provides a direct measure of the fraction of closed RCs. Therefore, an increase of  $q_p$  is not due to an increase of a photochemical rate constant, but simply to an increase of the concentration of open RCs which, by definition, are those exhibiting a lower fluorescence intensity. Moreover, any sample exhibits at any moment after any light condition a certain relative variable fluorescence  $0 \leq V \leq 1$  which is determined by many parameters; photochemical quenching (in terms of physics) is only one of them.

Both the naming of  $q_p$  and  $q_N$  and the symbols used, make the impression that they are describing complementary actions or mechanisms. This however is not at all the case.  $q_p$  is an expression which refers to one single state in which the fraction of open RCs can take only values between zero and unity. On the other hand,  $q_N$  is only defined by the extrema  $F_0$  and  $F_M$  (for all open and all closed RCs) at a particular state relative to the same expression obtained in the dark-adapted state. It is trivial that, if the quantum yield of primary photochemistry can be calculated only by the values  $F_0$  and  $F_M$  as  $\phi_{p_0} = 1 - F_0/F_M$ , any acrobatic expression which contains only the  $F_0$  and  $F_M$  can also be expressed in terms of  $\phi_{p_0}$ . The quantum yield of primary photochemistry however, is a function of  $k_N$  and  $k_p$  as  $\phi_{p_0}$  sample this may be because  ${}^1k_N$  is bigger,  ${}^4k_N$  is smaller,  ${}^4k_p$  is bigger or  ${}^1k_p$  is smaller, etc. This means that any change of  $q_N$  can be due to changes of  $k_p$  or/and  $k_N$ . The non-photochemical events can be described by non-photochemical de-excitation rate constants but not by the fashionable expression  $q_N = 1 - ({}^1F_V/{}^1F_0) / ({}^4F_V/{}^4F_0)$ .

#### *The meaning of the slope at the origin of the fast fluorescence rise*

The slope at the origin of the fluorescence rise  $(dF/dt)_0$  can be approximated by  $(\Delta F/\Delta t)_0$ ; the shorter the  $\Delta t$ , better is the approximation. This can be obtained with an instrument of a high time resolution (10  $\mu$ s or faster). The initial slope of the relative

variable fluorescence can then be expressed as the initial increment per ms, using, for example, the time interval of 250 or 100  $\mu\text{s}$ :

$$\begin{aligned} (dV/dt)_0 &\cong (\Delta V/\Delta t)_0 \\ &= \{ [\Delta F / (F_M - F_0)] / \Delta t \}_0 \\ &\cong 4 \cdot (F_{300\mu\text{s}} - F_{50\mu\text{s}}) / (F_M - F_{50\mu\text{s}}) \\ &\cong 10 \cdot (F_{150\mu\text{s}} - F_{50\mu\text{s}}) / (F_M - F_{50\mu\text{s}}) \end{aligned}$$

However, this expression corresponds to the maximal rate of the accumulation of the fraction of closed reaction centers,  $(dV/dt)_0 = (dB/dt)_0$ , and considering

- one  $Q_A$  per reaction centre,
- that one trapped exciton by an open RC triggers the reduction of  $Q_A$  to  $Q_A^-$ , and
- that the reoxidation of  $Q_A^-$  to  $Q_A$  is inhibited,

we can write:

$$\begin{aligned} (dV/dt)_0 &= (dB/dt)_0 \\ &= [d(Q_A^-/Q_{A(\text{total})})/dt]_0 \\ &= \text{flux of excitons trapped per RC} \\ &= TR_0/RC. \end{aligned}$$

Thus, in cases that the reoxidation of  $Q_A^-$  to  $Q_A$  is inhibited, as, for example, in the presence of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), the initial slope of the relative variable fluorescence  $(dV/dt)_0$  directly describes the trapping flux  $TR_0/RC$ . However, it has been shown (Strasser and Strasser, 1995) that when the reoxidation of  $Q_A^-$  to  $Q_A$  proceeds normally, the initial slope of the relative variable fluorescence has to be normalized by the value of  $V_j$  in order to describe the specific trapping flux  $TR_0/RC$ :

$$TR_0/RC = [(dV/dt)_0 / V_j] \cong 4 \cdot (F_{300\mu\text{s}} - F_{50\mu\text{s}}) / (F_{2\text{ms}} - F_{50\mu\text{s}})$$

#### *The area growth above the fast fluorescence rise*

Once a fluorescence rise from  $F_0$  to  $F_M$  is digitized and stored, the area growth between the measured fluorescence signal  $F_t$  and the maximal measured fluorescence can be calculated. The maximal area, i.e. the area up to the time  $t_{F_{\text{max}}}$  that  $F_t$  reaches  $F_M$ , denoted as "Area", is given by:

$$\text{Area} = \int_0^{t_{F_{\max}}} (F_M - F_t) dt$$

In order to compare different samples, the Area has to be normalized by  $F_v$ . The normalized expression is defined as:  $S_m \equiv \text{Area} / (F_M - F_0)$ . The parameter  $S_m$  expresses a work-integral; in other words, it is a measure of the energy needed to close all reaction centers. Subscript “m” stands for “multiple”, referring to the multiple turn-over in the closure of the reaction centers. The more the electrons from  $Q_A^-$  are transferred into the electron transport chain ET, the longer the fluorescence signals remain lower than  $F_M$  and the bigger  $S_m$  becomes. The smallest  $S_m$  corresponds to the case when every  $Q_A$  is reduced only once, as in the presence of DCMU, and it can then be denoted as  $S_s$  indicating single turn-over. Thereafter, the so-called turn-over number  $N$ , calculated as

$$N = \frac{S_m (\text{without DCMU, multiple turn-over})}{S_s (\text{with DCMU, single turn-over})}$$

indicates how many times  $Q_A$  has been reduced in the time span from 0 to  $t_{F_{\max}}$ .

If we consider an exponential fluorescence rise for the single turn-over situation, then the normalized area  $S_s$  would be inversely proportional to the initial slope of the relative variable fluorescence. However, as mentioned under “The meaning of the slope at the origin of the fast fluorescence rise”, the value of this slope can also be calculated in the absence of DCMU by dividing the actual initial slope of the relative variable fluorescence transient by  $V_j$  (Strasser and Strasser, 1995). Hence, the turn-over number can be given by a formula utilizing only data from the fluorescence transient of samples measured under physiological conditions, without the need of additional measurements in the presence of DCMU:

$$N = S_m \cdot [(dV/dt)_0] / V_j$$

#### *The time $t_{F_{\max}}$ to reach $F_M$*

The time to reach the maximal fluorescence intensity makes sense only if it can be measured accurately. A clear  $F_M$  has to appear in the fluorescence transient. Under these situations the ratio  $S_m/t_{F_{\max}}$  expresses the average redox state of  $Q_A$  in the time span from 0 to  $t_{F_{\max}}$  and, concomitantly, the average fraction of open reaction centers during the time needed to complete their closure:

$$S_m/t_{F_{\max}} = [Q_A/Q_{A(\text{total})}]_{\text{av}} = [1 - (Q_A^-/Q_{A(\text{total})})]_{\text{av}} = 1 - B_{\text{av}}$$

Therefore, this expression can be used for quantitation of the electron transport activity.

#### *The basic information obtainable from the fluorescence rise*

A fluorescence rise of high time resolution (10  $\mu\text{s}$  or faster) provides the following basic elements of information:

- ratio of the extrema ( $F_0/F_M$ )
- fluorescence intensity at any time ( $F_t$ )
- shape of the fluorescence kinetics ( $V_t$ )
- time to reach the maximal fluorescence ( $t_{F_{max}}$ ), and
- area above the fluorescence transient (Area).

Due to the typical shape of the fluorescence rise which shows the steps O, (K), J, I, P, the following information can easily be selected and used in screening tests:

- (1) ratio of the extrema,  $F_0/F_M$
- (2) normalized slope at the origin,  $(dV/dt)_0$
- (3) intermediate step,  $V_J$
- (4) intermediate step,  $V_I$
- (5) time to reach the maximal fluorescence,  $t_{F_{max}}$ , and
- (6) normalized area,  $S_m$ .

The information (1) to (3) refer to the structure and function of PSII only, while the information (4) to (6) refer to the activity of the electron transport beyond  $Q_A^-$  and to the activity of the photosynthetic metabolism. Therefore, it is possible to localize these two sites of responses of a sample to a physical or chemical stress from outside.

### The JIP-test: a tool for screening

Based on the analysis of how the data from the O-J-I-P fluorescence transient can be processed, a test has been developed and called the "JIP-test" after the steps of the transient. This test can be used as a tool for rapid screening of many samples providing adequate information about the structure, conformation and function of their photosynthetic apparatus (Strasser and Strasser, 1995; Strasser *et al.*, 1996).

We judged that it would be useful if this section on the JIP-test were not limited to a description of how the JIP-test can be applied, but integrated also the conceptual basis, even though some of the elements of this basis have already been analysed in the previous section.

From the data stored during the first second, the following values are selected to be used by the JIP-test for the calculation of several phenomenological and biophysical expressions leading to the dynamic description of a photosynthetic sample at a given physiological state:

- the maximal measured fluorescence intensity  $F_p$ , provided that the excitation intensity is high enough to permit the closure of all RCs so that  $F_p = F_M$ ;
- the fluorescence intensity at  $50 \mu s$ , considered to be  $F_0$ , i.e. the intensity when all RCs are open;
- the fluorescence intensity at  $150 \mu s$ , at  $300 \mu s$ , at  $2 ms$  (denoted as  $F_J$ ) and at  $60 ms$  (denoted as  $F_I$ );
- the time to reach  $F_M$   $t_{F_{max}}$ ; and
- the area between the fluorescence transient and the line  $F = F_M$ .

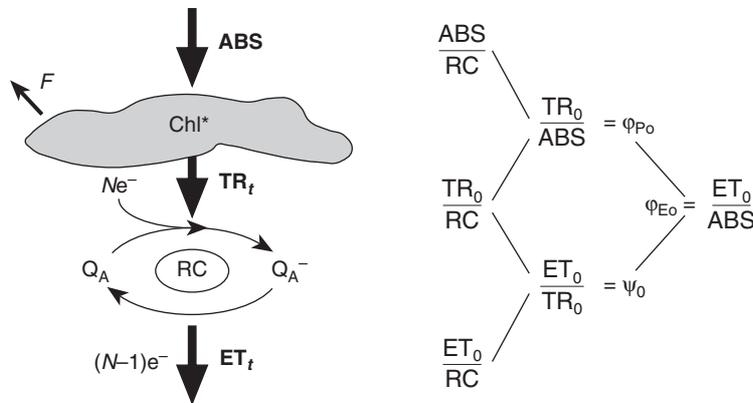


Figure 25.12 A highly simplified working model of the energy fluxes in the photosynthetic apparatus.

A highly simplified working model of the energy fluxes in a photosynthetic apparatus is shown in Figure 25.12 (from Strasser and Strasser, 1995). Based on the theory of energy fluxes in biomembranes, formulae for the specific energy fluxes (per reaction centre RC) and the phenomenological energy fluxes (per excited cross-section CS), as well as for the flux ratios or yields, have been derived using the experimental values provided from the JIP-test. The constellation of their values at any instant can be considered as expressing the behaviour of the system (Krüger *et al.*, 1997). ABS refers to the flux of photons absorbed by the antenna pigments Chl\*. Part of this excitation energy is dissipated, mainly as heat and less as fluorescence emission F, and another part is channelled as trapping flux TR to the reaction centre RC and converted to redox energy by reducing the electron acceptor Q<sub>A</sub> to Q<sub>A</sub><sup>-</sup>, which is then reoxidized to Q<sub>A</sub> thus creating an electron transport ET that leads ultimately to CO<sub>2</sub> fixation.

The specific energy fluxes at time zero (at the onset of excitation) ABS/RC, TR<sub>0</sub>/RC and ET<sub>0</sub>/RC, can be derived from the experiments as shown below. The maximum quantum yield of primary photochemistry (note that trapping refers to the energy flux leading to photochemistry) TR<sub>0</sub>/ABS ≡ φ<sub>Po</sub>, the efficiency that a trapped exciton can move an electron further than Q<sub>A</sub><sup>-</sup> into the electron transport chain ET<sub>0</sub>/TR<sub>0</sub> ≡ ψ<sub>0</sub>, or the probability that an absorbed photon will move an electron into the electron transport chain ET<sub>0</sub>/ABS ≡ φ<sub>Eo</sub>, are directly related to the three fluxes, as the ratios of any two of them. TR/RC expresses the rate by which an exciton is trapped by the RC resulting in the reduction of Q<sub>A</sub> to Q<sub>A</sub><sup>-</sup>. The maximal value of this rate is given by TR<sub>0</sub>/RC, because at time zero all RCs are open. The link of TR<sub>0</sub>/RC with the experimental data is derived as follows. If the reoxidation of Q<sub>A</sub><sup>-</sup> would be blocked, as happens in DCMU-treated samples, TR<sub>0</sub>/RC would be given by the normalization of the initial slope of the fluorescence induction curve (between 50 and 300 μs or 50 and 150 μs) on the maximal variable fluorescence F<sub>V</sub> = F<sub>M</sub> - F<sub>0</sub>. This normalized value is denoted as M<sub>0,DCMU</sub>. However, if the reoxidation of Q<sub>A</sub><sup>-</sup> is not blocked, the normalized value of the initial slope, M<sub>0</sub>, indicates the net rate of closure of the RCs, where trapping increases the number of closed centers and electron transport decreases it:

$$M_0 = TR_0/RC - ET_0/RC \quad (25.12)$$

A thorough investigation carried out in our laboratory (Strasser and Strasser, 1995) revealed clearly that  $M_0$  in DCMU-treated samples can be simulated by the amplification of the measured  $M_0$  in samples without DCMU by a factor reciprocal to  $V_j$ . Thus,

$$TR_0/RC = M_{0,DCMU} = M_0/V_j \quad (25.13)$$

From equations 25.12 and 25.13 we get:

$$\begin{aligned} ET_0/RC &= TR_0/RC - M_0 \\ &= (M_0/V_j) - M_0 \\ &= (M_0/V_j) \cdot (1 - V_j) \\ &= (TR_0/RC) \cdot (1 - V_j) \end{aligned} \quad (25.14)$$

Hence,

$$\Psi_0 \equiv ET_0/TR_0 = 1 - V_j \quad (25.15)$$

and

$$\varphi_{E_0} \equiv ET_0/ABS = (TR_0/ABS) \cdot (ET_0/TR_0) = \varphi_{P_0} \cdot \Psi_0 \quad (25.16)$$

where  $\varphi_{P_0}$  is calculated from the values at the extrema  $F_0$  and  $F_M$  of the fluorescence transient:

$$\varphi_{P_0} = (F_M - F_0) / F_M = 1 - (F_0/F_M) \quad (25.17)$$

Based on equations (25.16) and (25.17), equation (25.15) can now be written as:

$$\varphi_{E_0} = [1 - (F_0/F_M)] \cdot (1 - V_j) \quad (25.18)$$

Concerning the expression  $ABS/RC$ , it is derived as follows:

$$TR_0/RC = (TR_0/ABS) \cdot (ABS/RC) = \varphi_{P_0} \cdot (ABS/RC) \quad (25.19)$$

$$ABS/RC = (TR_0/RC) / \varphi_{P_0} = (M_0/V_j) / [1 - (F_0/F_M)] \quad (25.20)$$

It has to be pointed out that  $TR_0/RC$  expresses the initial rate of the closure of RCs as a fractional expression over the total number of RCs that can be closed. We point this out clearly, because it is possible that under stress conditions some RCs are inactivated in the sense of being transformed to quenching sinks (Krause *et al.*, 1990) without reducing  $Q_A$  to  $Q_A^-$ . In such a case,  $TR_0/RC$  still refers only to the active ( $Q_A$  to  $Q_A^-$ -reducing) centers. The same is valid for the other two specific fluxes, since their derivation is based on  $TR_0/RC$ .

The phenomenological fluxes are, accordingly,  $ABS/CS$ ,  $TR_0/CS$  and  $ET_0/CS$ , where  $CS$  stands for the excited cross-section of the tested sample. The value of the initial fluorescence  $F_0$  has been proposed to serve as a measure (in arbitrary units) of the phenomenological absorption flux  $ABS/CS$  (Strasser and Strasser, 1995). However, since the initial fluorescence value is affected by conformational changes, care should be taken (Krüger *et al.*, 1997) to use the value  $F_0^{\text{dark}}$  that the sample exhibits while being in its dark-adapted state:

$$ABS/CS_0 = F_0^{\text{dark}} \quad (25.21)$$

$TR_0/CS_0$  and  $ET_0/CS_0$  can then be calculated, in the same arbitrary units:

$$TR_0/CS_0 = \varphi_{p_0} \cdot F_0^{\text{dark}} = [1 - (F_0/F_M)] \cdot F_0^{\text{dark}} \quad (25.22)$$

$$ET_0/CS_0 = \varphi_{E_0} \cdot F_0^{\text{dark}} = [1 - (F_0/F_M)] \cdot (1 - V_j) \cdot F_0^{\text{dark}} \quad (25.23)$$

The expression  $RC/CS_0$  (active RCs per excited cross-section) for the concentration of the reaction centers is easily derived using equations (25.20) and (25.21):

$$RC/CS_0 = (ABS/CS) / (ABS/RC) = \{ F_0^{\text{dark}} \cdot [1 - (F_0/F_M)] \} / (M_0/V_j) \quad (25.24)$$

The value of the maximal fluorescence  $F_M$  can also serve as a measure (in arbitrary units) of the phenomenological absorption flux, provided that the same care is taken to avoid the interference of conformational changes. In order to distinguish the two ways of calculation, the following notations are used:  $ABS/CS_0 = F_0^{\text{dark}}$  and  $ABS/CS_M = F_m^{\text{dark}}$ .

All these formulae for the JIP-test are summarized in Table 25.5.

Based on the theory of energy fluxes in biomembranes, expressions relating the photochemical rate constant  $k_p$  and the nonphotochemical rate constant  $k_N$  summing up  $k_H$  (for heat dissipation),  $k_F$  (for fluorescence emission) and  $k_X$  (for energy migration to PSI) with the fluorescence values  $F_0$  and  $F_M$  have been derived (see Havaux *et al.*, 1991):

$$k_N = (ABS/CS) \cdot k_F \cdot (1/F_M) \quad (25.25)$$

$$k_N + k_p = (ABS/CS) \cdot k_F \cdot (1/F_0) \quad (25.26)$$

$$k_p = (ABS/CS) \cdot k_F \cdot \{ (1/F_0) - (1/F_M) \} \quad (25.27)$$

Note that

$$\varphi_{p_0} = 1 - (F_0/F_M) = k_p / (k_p + k_N) \quad (25.28)$$

### **The presentation of the JIP-test data**

A screening test has to be able to present a large amount of data in an integrated way, in order to allow a clear approach to the situation under study. As an example we show

Table 25.5 Summary of the JIP-test formulae, using data extracted from the fast fluorescence transient

<i>Extracted and technical fluorescence parameters</i>	
$F_0$	= $F_{50\mu s}$ , fluorescence intensity at 50 $\mu s$
$F_{150}$	= fluorescence intensity at 150 $\mu s$
$F_{300}$	= fluorescence intensity at 300 $\mu s$
$F_J$	= fluorescence intensity at the J-step (at 2 ms)
$F_M$	= maximal fluorescence intensity
$t_{fmax}$	= time to reach $F_M$ , in ms
$V_J$	= $(F_{2ms} - F_0) / (F_M - F_0)$
Area	= area between fluorescence curve and $F_M$
$(dV/dt)_0$ or $M_0$	= $4 \cdot (F_{300} - F_0) / (F_M - F_0)$
$S_m$	= Area / $(F_M - F_0)$
$B_{av}$	= $1 - (S_m / t_{fmax})$
$N$	= $S_m \cdot M_0 \cdot (1 / V_J)$ turn-over number $Q_A$
<b>Quantum efficiencies or flux ratios</b>	
$\Phi_{Po}$ or $TR_0 / ABS$	= $(1 - F_0) / F_M$ or $F_v / F_M$
$\Phi_{Eo}$ or $ET_0 / ABS$	= $(1 - F_0 / F_M) \cdot \Psi_0$
$\Psi_0$ or $ET_0 / TR_0$	= $1 - V_J$
<b>Specific fluxes or specific activities</b>	
$ABS / RC$	= $M_0 \cdot (1 / V_J) \cdot (1 / \Phi_{Po})$
$TR_0 / RC$	= $M_0 \cdot (1 / V_J)$
$ET_0 / RC$	= $M_0 \cdot (1 / V_J) \cdot \Psi_0$
$DI_0 / RC$	= $(ABS / RC) - (TR_0 / RC)$
<b>Phenomenological fluxes or phenomenological activities</b>	
$ABS / CS_0$	= $F_0$ or other useful expression *
$TR_0 / CS_0$	= $\Phi_{Po} (ABS / CS_0)$
$ET_0 / CS_0$	= $\Phi_{Po} \cdot \Psi_0 \cdot (ABS / CS_0)$
$DI_0 / CS_0$	= $(ABS / CS_0) - (TR_0 / CS_0)$
$RC / CS_0$	= $\Phi_{Po} \cdot (V_J / M_0) \cdot F_0$ *

\* when expressed per  $CS_M$ ,  $F_0$  is replaced by  $F_M$

here how the effect of a mild heat-treatment (10 min at 40°C or 44°C) on the behaviour of pea leaves can be presented in such an integrated way. The JIP-test of one single excitation of 1 sec light was used for both the heat-treated samples and the non-treated samples (25°C) which served as the control. The fast fluorescence transients are shown in Figure 25.13. All expressions were calculated according to the equations of Table 25.5 and the calculated values are summarized in Tables 25.6 and 25.7, both as absolute values and relative to the corresponding values of the control. All measured information (all obtained from one single sample and an experimental excitation time of one second only) is summarized in two types of presentation: the spider-plot presentation and the energy pipeline model. The experiment shows clearly most of the well-known responses to a mild heat treatment.

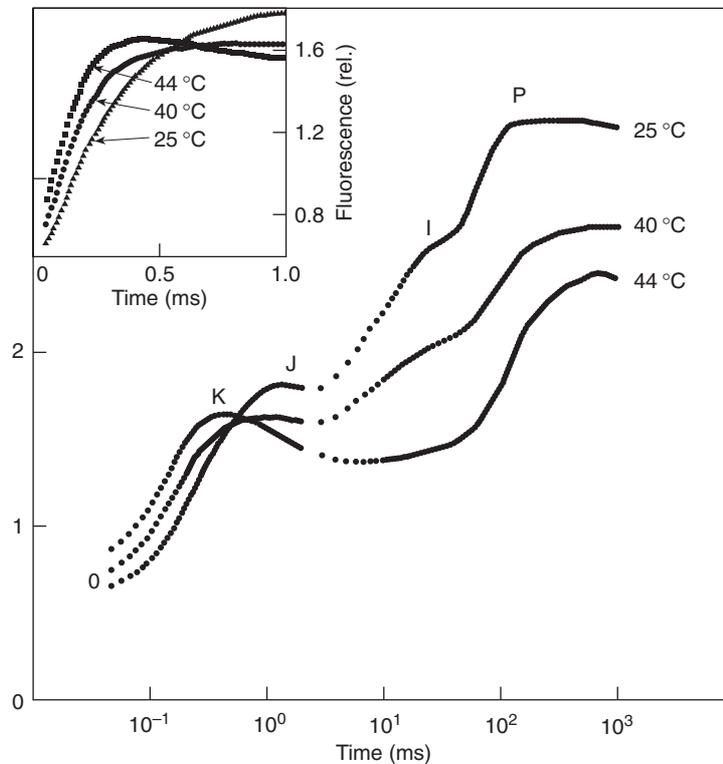


Figure 25.13 The fast fluorescence rise (measured with the PEA instrument) in pea leaves subjected for 10 min to a heat treatment at 40°C and 44°C. The fast fluorescence rise of the control sample (25°C) is also shown. Insert shows the same curves but plotted on linear time scale up to 1 ms.

#### The spider-plot presentation

The relative values (relative to the corresponding value of the control, which thus become equal to unity) of selected expressions, such as the specific fluxes ( $ABS/RC$ ,  $TR_0/RC$  and  $ET_0/RC$ ), the flux ratio  $TR_0/ABS = \phi_{po}$ , and the density of the active reaction centers per excited cross-section ( $RC/CS$ ), can be plotted using a spider-plot presentation (Figure 25.14). This is a multiparametric description of structure and function of each photosynthetic sample, presented by an octagonal line. This type of presentation provides a direct visualization of the behaviour of a sample and thus facilitates the comparison of plant material as well as the classification of the effect of different environmental stressors on it in terms of the modifications it undergoes to adapt to new conditions.

#### Energy pipeline models

The derived parameters can also be visualized by means of an energy pipeline model of the photosynthetic apparatus (Strasser, 1987; Strasser *et al.*, 1996; Krüger *et al.*, 1997). This is a dynamic model in which the value of each energy flux, either changing as a

Table 25.6 The experimental expressions of the JIP-test and their calculated values from the data of Figure 25.13. Pea (*Pisum sativum*) leaves were heated at 40°C and 44°C for 10 min. Fluorescence measurements were performed immediately after heat treatment at room temperature. Numbers in parentheses express the normalized values over the control.

<i>Pisum sativum</i>	Control		10 min heat treatment			
	25°C		40°C		44°C	
<b>F extremes</b>						
$F_0$	663	(1)	759	(1.14)	873	(1.32)
$F_M$	3357	(1)	2734	(0.81)	2465	(0.73)
$F_V / F_0$	4.06	(1)	2.60	(0.64)	1.82	(0.45)
<b>F dynamics</b>						
$V_j$	0.43	(1)	0.44	(1.04)	0.49	(1.14)
$V_i$	0.73	(1)	0.65	(0.89)	0.37	(0.50)
$(dV/dt)_0$	0.97	(1)	1.42	(1.46)	1.83	(1.89)
<b>Areas</b>						
$\log S_m$	1.37	(1)	1.68	(1.22)	2.01	(1.47)
$S_m / t_{F_{max}}$	98.9	(1)	65.9	(0.67)	150	1.52
$\log N$	1.73	(1)	2.19	(1.27)	2.59	(1.50)

Table 25.7 The specific and phenomenological expressions of the JIP-test and their calculated values from the data of Figure 25.13. Pea (*Pisum sativum*) leaves were heated at 40°C and 44°C for 10 min. Fluorescence measurements were performed immediately after heat treatment at room temperature. Numbers in parentheses express the normalized values over the control.

<i>Pisum sativum</i>	Control		10 min heat treatment			
	25°C		40°C		44°C	
<b>Flux per RC</b>						
ABS/RC = AS	2.84	(1)	4.45	(1.57)	5.85	(2.06)
TR <sub>0</sub> /RC	2.28	(1)	3.21	(1.41)	3.78	(1.66)
ET <sub>0</sub> /RC	1.31	(1)	1.79	(1.37)	1.94	(1.48)
<b>Flux-ratio = Yield</b>						
TR <sub>0</sub> /ABS = $\varphi_{Po}$	0.80	(1)	0.72	(0.90)	0.65	(0.80)
ET <sub>0</sub> /TR <sub>0</sub> = $\psi_0$	0.57	(1)	0.56	(0.97)	0.51	(0.89)
ET <sub>0</sub> /ABS = $\varphi_{Eo}$	0.46	(1)	0.40	(0.87)	0.33	(0.72)
<b>Density of RCs</b>						
RC/CS <sub>0</sub> = (Chl / CS) : (Chl / RC)						
= F <sub>0</sub> / AS	234	(1)	171	(0.73)	149	(0.64)
RC/CS <sub>M</sub> = (Chl / CS) : (Chl / RC)						
= F <sub>M</sub> / AS	1183	(1)	615	(0.52)	421	(0.36)
<b>Activities</b>						
ABS/CS <sub>0</sub> = F <sub>0</sub>	663	(1)	759	(1.14)	873	(1.32)
TR <sub>0</sub> /CS <sub>0</sub> = F <sub>0</sub> • $\varphi_{Po}$	532	(1)	548	(1.03)	564	(1.06)
ET <sub>0</sub> /CS <sub>0</sub> = F <sub>0</sub> • $\varphi_{Po}$ • $\psi_0$	306	(1)	306	(1.00)	290	(0.95)
ABS/CS <sub>M</sub> = F <sub>M</sub>	3357	(1)	2734	(0.81)	2465	(0.73)
TR <sub>0</sub> /CS <sub>M</sub> = F <sub>M</sub> • $\varphi_{Po}$	2694	(1)	1975	(0.73)	1592	(0.59)
ET <sub>0</sub> /CS <sub>SM</sub> = F <sub>M</sub> • $\varphi_{Po}$ • $\psi_0$	1549	(1)	1103	(0.71)	819	(0.53)

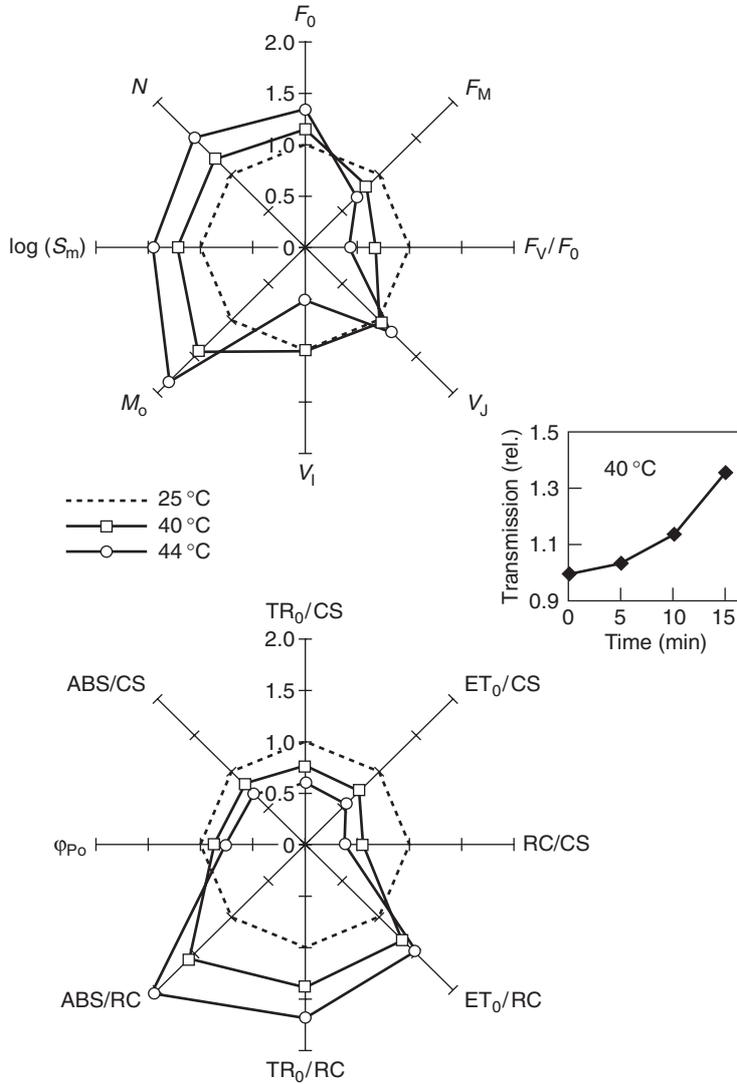


Figure 25.14 An example of a spider-plot presentation of a constellation of selected parameters quantifying the behaviour of PSII of pea leaves exposed for 10 min to 40°C (□) or 44°C (○), relative to that of pea leaves at 25°C (regular octagon, ----). Top: technical spider-plot showing normalized experimental expressions. Bottom: flux spider-plot showing specific and phenomenological fluxes which have been calculated from the values of the technical spider-plot according to the equations given in Table 25.5. The effect of heat treatment on the light transmission of the tested leaves is shown in the insert.

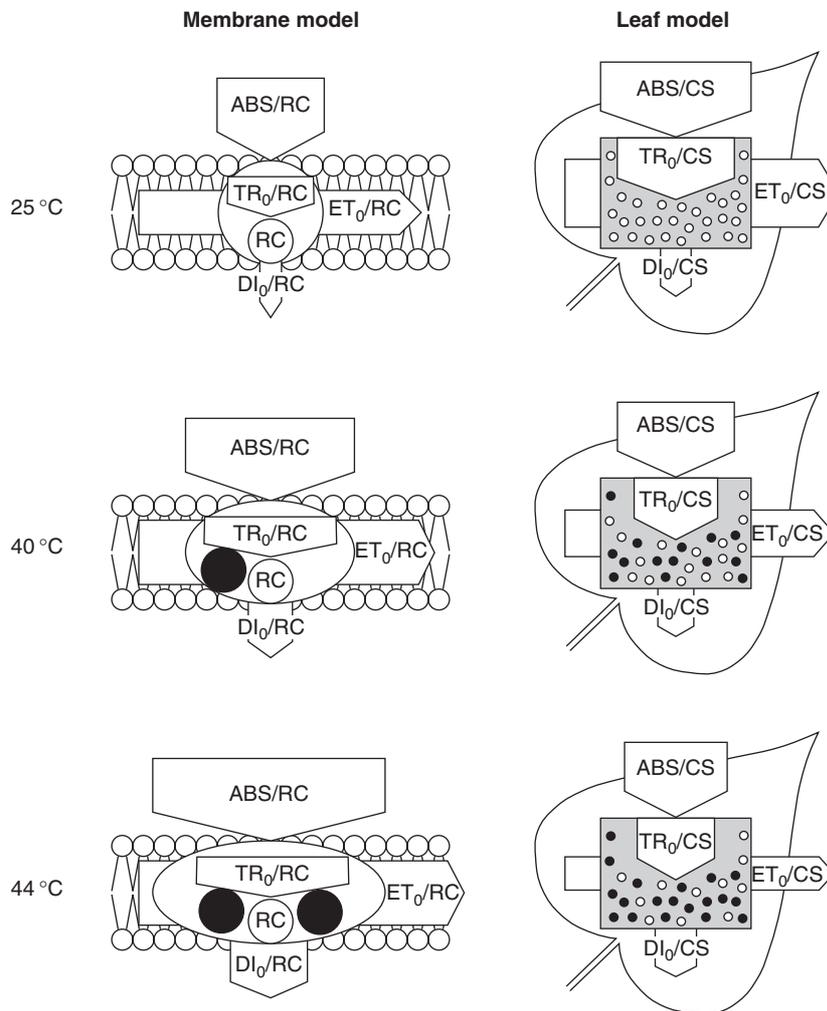


Figure 25.15 Pipeline models for specific fluxes (membrane model) or phenomenological fluxes (leaf model).

function of time or modified by the different imposed environmental conditions, is expressed by the appropriately adjusted width of the corresponding arrow.

For each sample and/or state of the sample, two types of models can be presented (Figure 25.15); the one refers to the reaction centre in the membrane and thus deals with the *specific energy fluxes* (per RC) and the other refers to the excited cross-section of a leaf and thus deals with the *phenomenological energy fluxes* (per CS). The flux of dissipated excitation energy at time zero ( $DI_0 = ABS - TR_0$ ) is also shown both per RC and per CS. The membrane model includes also a demonstration of the average “antenna size”, which follows the value of the  $ABS/RC$ . This value expresses the total absorption of PSII antenna chlorophylls divided by the number of active (in the sense of  $Q_A$  reducing) reaction centres. Therefore, the antenna of inactivated reaction centres (non- $Q_A$  reducing, here due to the depletion of the oxygen-evolving side) are mathematically

added to the antenna of the active reaction centres. In the leaf model the active reaction centers per cross-section (RC/CS) are indicated by open circles and those that have been inactivated are indicated by closed circles.

Upon heating, the data show the following:

- Phenomenologically per excited cross-section CS (i.e., per active measured leaf area):
  - Decrease of electron transport per excited cross-section ( $ET_0/CS$ ) due to the inactivation of reaction centre complexes (here due to the inactivation of the oxygen-evolving system).
  - Decrease of the density of active reaction centers RC/CS (indicated as open circles in Figure 25.15).
  - Increase of the energy dissipation per excited cross-section  $DI_0/CS$ .
  - Decrease of the energy absorbed per excited cross-section  $ABS/CS$ . This finding has been confirmed by independent parallel transmission measurements which showed an increase of the transmitted light through the leaf (insert in Figure 25.14).
- Specifically per active reaction centre RC:
  - Average absorption per active reaction centre increases ( $ABS/RC$ ) due to the inactivation of some RCs.
  - The ratio of total dissipation to the amount of active RCs increases ( $DI_0/RC$ ) due to the high dissipation of the inactive RCs.
  - Electron transport per active reaction centre increases due to a thermal activation of the dark reactions.

### Concluding remarks

In this overview we have discussed some of the properties of the chlorophyll fluorescence induction curves and showed how they can be utilized to derive information about the behaviour of PSII. However, the recorded fluorescence transients carry much more information than those used by the JIP-test. The whole of this information can be utilized by means of the powerful methods of numerical simulations (Stirbet and Strasser, 1995, 1996; Stirbet *et al.*, 1998). The application of these methods is being further explored in our laboratory.

The fluorescence kinetic is so rich in information that since its discovery in 1931 new properties are continuously being detected. New instrumentation has made it possible to measure fast changes in a way that one can follow the electron transfer from the water splitting side to  $Q_A$ , then to  $Q_B$  and later to plastoquinone. As this transfer is very sensitive to stressors and highly dependent on the need of electrons for metabolism, PSII fluorescence can become a biosensing device for stress detection in plants. For such detection, it is very useful to establish and apply tests that can screen many samples in a short time. At a second stage, more time-consuming and specific tests can be made on selected samples. In the future, the newer fluorescence imaging techniques and numerical simulations will have to be calibrated by accurate kinetic tests like the JIP-test.

Basic fluorescence understanding combined with the JIP-test are a tool to analyse any plant material in any situation, even by non-fluorescence specialists. We hope that

many young scientists will try the JIP-test presented in this report to probe photosynthesis.

### Remarks

While this paper has been processed we derived a so called performance index, PI, of any photosynthetic sample. For its derivation see Strasser *et al.* (1999) and Srivastava *et al.* (1999).

This index groups the bifurcations of the energy cascades:

$$PI = \frac{ABS}{CS} \cdot \frac{RC}{ABS} \cdot \frac{TR}{DI} \cdot \frac{ET}{dQ_A^- / dt}$$

or in experimental terms, based on the cross section or leaf area

$$PI_{CS_0} = F_0 \cdot \frac{RC}{ABS} \cdot \frac{\varphi_{P_0}}{1 - \varphi_{P_0}} \cdot \frac{\psi_0}{1 - \psi_0}$$

or

$$PI_{CSM} = F_M \cdot \frac{RC}{ABS} \cdot \frac{\varphi_{P_0}}{1 - \varphi_{P_0}} \cdot \frac{\psi_0}{1 - \psi_0}$$

or based on the equal absorption

$$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi_{P_0}}{1 - \varphi_{P_0}} \cdot \frac{\psi_0}{1 - \psi_0}$$

To present the different components we defined a driving force of the primary photosynthetic reactions as

$$DF_{(x)} = \log PI_{(x)} \text{ which is the sum of the logarithms of the components above.}$$

For many situations the expressions  $PI_{(x)}$  and  $DF_{(x)}$  appeared to be a very sensitive index for any stress and therefore, a practical index to use for physiological, environmental and biotechnological screenings.

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