7

Chlorophyll Fluorescence in Plant Biology

Amarendra Narayan Misra[1,2], Meena Misra[1,2] and Ranjeet Singh[1,2]
[1]*Post-Graduate Department of Biosciences & Biotechnology, Fakir Mohan University, Balasore,*
[2]*Centre for Life Sciences, School of Natural Sciences, Central University of Jharkhand, Ratu-Lohardaga Road, Brambe, Ranchi, India*

1. Introduction

1.1 Chlorophyll fluorescence: Basics

Several molecules absorb light energy which they emit after a time difference (lifetime) as radiation energy. Molecules remain at a low energy level or the ground electronic singlet state (S_o) or the lowest vibrational level at room temperature (Noomnarm and Clegg, 2009). On absorption of a photon, the molecule is excited from S_o to the first electronic excited singlet state S_1 within < 10-15 s^{-1} (Figure 1). These molecules can also be transferred to higher energy levels (S_2 to S_n) also. These excited state molecules can relax to the S_1 electronic state via vibrational relaxation within 10-12 s^{-1}. The molecule will ultimately relax to the S_o state through photon emission, which is called fluorescence emission. Also here, the energy of the emitted photon must equal the changes in the energy levels.

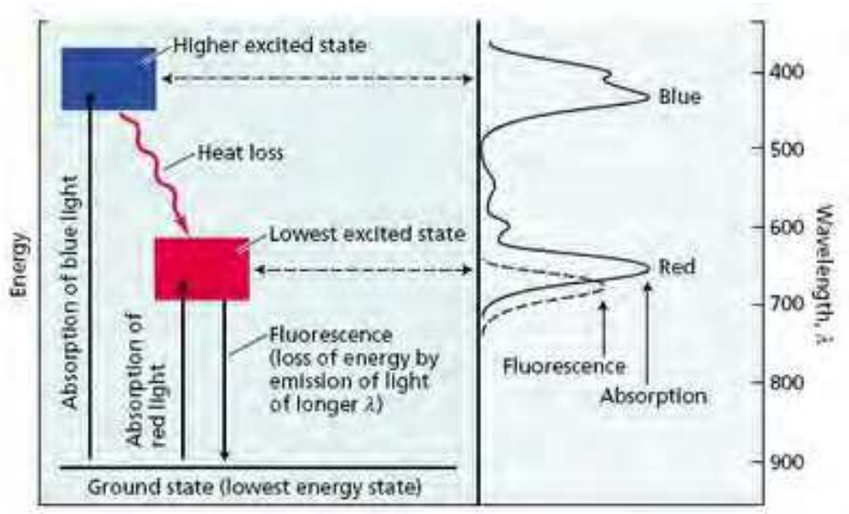


Fig. 1. The basic principles of excitation and deexcitation phenomena and the differences between excitation (absorption) spectra and emission (fluorescence) spectra of light absorbing pigment molecules (Chlorophyll) in green plants.

The molecular excitation follows the principle: $\Delta E = hv$, with ΔE, energy difference between ground and excited state; *h*, Planck quantum; and *v*, frequency of radiation (Rabinowitch & Govindjee, 1969; Kumke & Löhmannsröben, 2009).

Light energy is absorbed by chlorophyll, carotenoids and other pigment molecules present in the photosynthetic antenna molecules present in the thylakoid membranes of green plants (Strasser et al., 2000, 2004; Govindjee, 2004; Maxwell and Johnson, 2000; Falkowski & Raven, 2007). Absorption of a photon raises a chlorophyll *a* molecule to its lowest singlet excited state, for which three internal decay pathways exist: fluorescence, in which the molecule returns to the ground state with the emission of radiation; internal conversion, in which the energy of the molecule is converted into vibrational energy; and intersystem crossing, in which the singlet state is converted to the triplet state (Figure 2). If certain other molecules are present along with the chlorophyll, external decay pathway(s) may also become available in addition to the internal decay pathways. Such external pathways facilitate the transfer of energy to a molecule with a similar energy gap or the transfer of an electron to or from another molecule, such as in excitation energy transfer in light-harvesting antennae and charge separation in photochemical reaction centers, respectively. All of these downward processes competitively contribute to the decay of the chlorophyll excited state. Accordingly, an increase in the rate of one of these processes would increase its share of the decay process and lower the fluorescence yield (φf). The quantum yield of chlorophyll fluorescence from the photosynthetic apparatus is therefore 0.6-3%, while chlorophyll *a* in an organic solvent exhibits a high fluorescence yield of approximately 30% (Latimer et al., 1956; Trissl et al., 1993). Oxygenic photosynthesis is endowed with the unique property of a fluorescence emission. Light energy that is absorbed by chlorophyll in a photosynthetic systems can undergo three fates: a) it can be used to drive photosynthesis (photochemistry), b) it can be dissipated as heat or c) it can be re-emitted as red fluorescence (Figure 2). These three processes occur in competition. Since the sum of rate constants is constant, any increase in the efficiency of one process will result in a decrease in the yield of the other two. Therefore, determining the yield of chlorophyll fluorescence will give information about changes in the efficiency of photochemistry and heat dissipation (Figure 2).

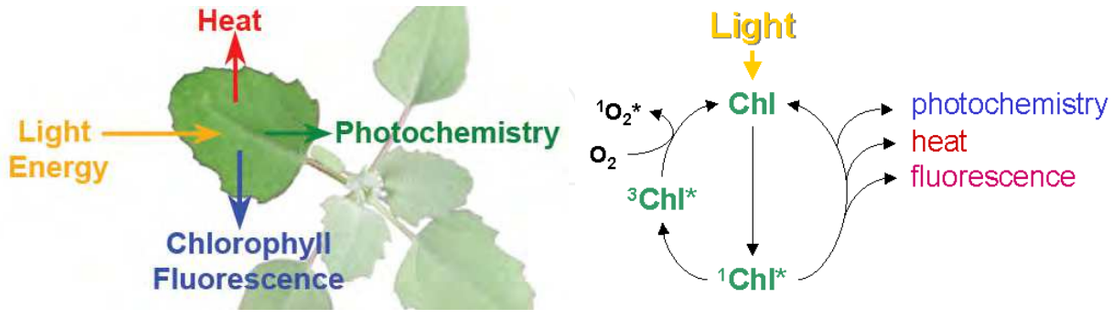


Fig. 2. The origin of chlorophyll fluorescence: basic aspects.

The oxygenic photosynthesis involves two light reactions operating simultaneously at photosystem (PS) II and PSI reaction centers (Figure 3). The light energy absorbed by the light harvesting antenna (LHC) pigments distribute the energy to the two photosystems, used to oxidize water to oxygen, reduce NADP+, and produce ATP (Rabinowitch & Govindjee, 1969; Blankenship, 2002; Falkowski & Raven, 2007). Most of the chlorophyll a

fluorescence, at room temperature, originates in the antenna complexes of PSII and originate as fluorescence emission at 685nm (F685) (Govindjee, 2004). The absorption of photons by antenna molecules is a very fast process and occurs within femtoseconds, leading to the formation of excited chlorophylls (Chl*). The main function of the antenna (LHC) is to transfer excitation energy to the photosynthetic reaction centers leading to photochemistry. But a part of the absorbed light energy is dissipated as heat and is emitted as fluorescence (Figure 3). Primary charge separation occurs in PSI and PSII reaction centers involving P700 and P680, respectively. Photochemistry takes place within picoseconds, and further reactions proceed independent of the presence of light (Stirbet & Govindjee, 2011). The characteristic of fluorescence emission is determined by the absorbing pigment molecules, the excitation energy transfer, and the orientation of the fluorescing pigments in the photosynthetic membrane. Besides these characteristics, fluorescence is also affected by the redox state of the donors and acceptors of photosystems, and thylakoid stacking etc. (Strasser et al. 2005). Although fluorescence measurements are indicators of indirect effects, still fluorescence is widely used as a luminescence signature for wide array of photosynthetic events and alterations in the photosynthetic systems. There are different types of fluorescence measurements used in plant biology and photosynthesis, which are described below. Depending on the type of study and the suitability of the photosynthetic system, different fluorescence techniques are used. The analysis of these fluorescence curves or images and its analysis gives an insight to the photosynthetic energy transducing or pigment protein orientation in the photosynthetic systems.

2. Types of chlorophyll fluorescence

Chlorophyll *a* fluorescence is a highly versatile tool, not only for researchers studying photosynthesis, but also for those working in broader fields related to biophysics, biochemistry and physiology of green plants. Chlorophyll fluorescence analysis is sensitive, non-invasive, and relatively simple. With the advent of different instrumental techniques and time resolved spectroscopy, fluorometry developed into various types with timescale of signal capturing. The fluorescence measurements, that are conventionally used, are

i. Room temperature fluorescence (Rabinowitch & Govindjee, 1969) ,
ii. Low temperature fluorescence (77K fluorescence) (Rabinowitch & Govindjee, 1969),
iii. Fluorescence temperature curve, (Ilik et al., 2003),
iv. Variable Chl a fluorescence, differing in the manner by which the photochemistry is saturated (e.g., shutterless and LED-based instruments) for direct fluorometry:
    a. fast Chl fluorescence or plant efficiency analyser (PEA) (Strasser & Govindjee, 1991; 1992),
    b. pulse amplitude modulation, PAM, fluorometry (Schreiber et al., 1986; Schreiber, 2004),
    c. the pump and probe (P & P) fluorometry (Mauzeralla, 1972; Falkowski et al., 1986),
    d. the fast repetition rate (FRR) fluorometry (Kolber et al. 1998),
    e. the pump during probe (PDP) fluorometry (Olson et al., 1996), and several others that are functionally similar, such as
    f. the fluorescence induction and relaxation (FIRe) technique (Gorbunov & Falkowski, 2005),
    g. the background irradiance gradient single turnover (BIG-STf) fluorometry (Johnson, 2004), and
    h. advanced laser fluorometry (ALF) (Chekalyuk & Hafez, 2008).

However, the working principle and the phenomenon for analysis are similar for these instruments. In the present chapter we describe the commonly used room temperature fluorescence, low temperature or 77 K fluorescence, fast Chl fluoresce and PAM fluorescence. The other methods are useful but are not discussed due to their specialized use in various fields. However, the techniques and principles described here are routinely used in plant biology at present.

2.1 Room temperature fluorescence

Under a physiological state of active chloroplasts in green plants at room temperature, chlorophyll fluorescence emission is a net result of heat dissipation, stimulation of dark reduction of plastoquinone, and increased cyclic electron flow to light, also increases the leakage of electrons from the thylakoid, there may be a deactivation of Rubisco (ribulose 1,5 bi-phosphatecarboxylase- oxygenase), and the generation of reactive oxygen species such as the superoxide anion (O_2^-) and H_2O_2. The chlorophyll fluorescence emission spectra is taken as a measure of the amount of chlorophyll content in the green plants (Buschmann, 2007). There are two maxima for Chl fluorescence at room temperature, (i) in the red region at 685 nm emitted by PS II and (ii) in the far-red region at 720-740 nm emitted by PS I. At higher chlorophyll concentrations, chlorophyll fluorescence is mainly detected in the range of 720-740 nm. But the re-absorption of the emitted red fluorescence by the chlorophyll in PS II results in a strong fluorescence emission band at 685 nm (Figure 4). The technique and the instrumentation are simple. The fluorescence emission is measured at right angle (90°) or 45° to the excitation beam of blue or red wavelength band of visible light. However, when cooled to liquid nitrogen temperature (77K) the fluorescence emission at 685nm, 695nm and 735nm can be resolved separately and can be analysed (see the section 77K fluorescence).

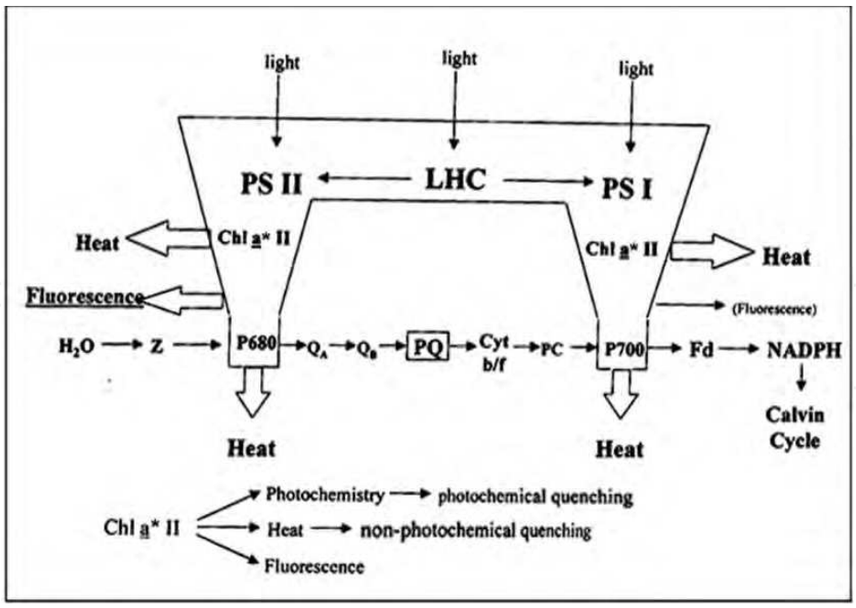


Fig. 3. Schematic illustration of primary conversion in photosynthesis which governs *in vivo* chlorophyll fluorescence yield. Variable fluorescence originates almost exclusively from PSII. Maximal fluorescence yield is lowered by photochemical charge separation and dissipation.

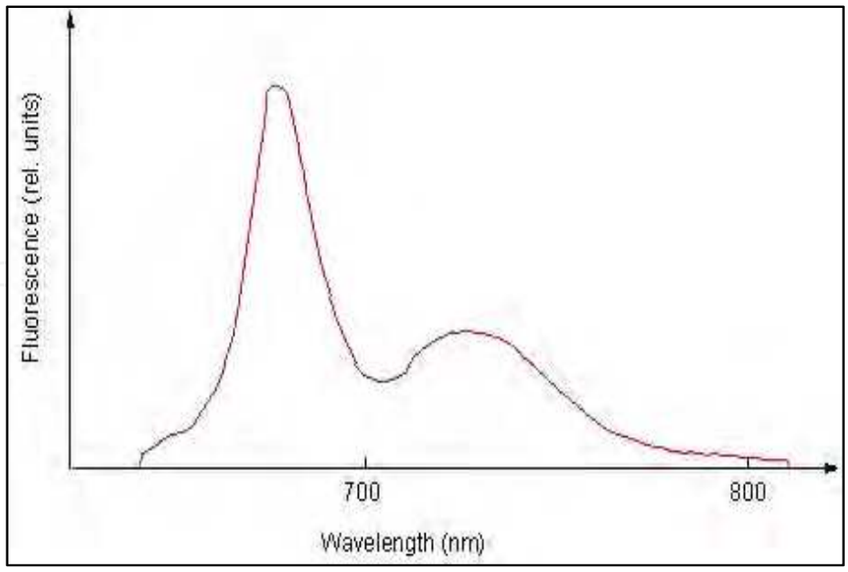


Fig. 4. A typical room temperature fluorescence emission by green to leaf.

2.2 77K fluorescence

The fluorescence at liquid-nitrogen temperature from algal cells and isolated thylakoid membranes to show a distinct spectral bands at approximately 685, 695, and 735 nm (Murata et al., 1966; Boardman et al., 1966; Govindjee et al., 1967). The two bands at approximately 685 and 695 nm corresponded to fluorescence emitted from Chl in PSII, while the band at 735 (usually a broad band between 715–740 nm) correspond to the fluorescence from the Chl in PSI (Figure 5). This study led to the discovery of state transitions, which is a regulatory mechanism for balancing the distribution of light energy between PSI and PS II. When algal cells were illuminated with light wavelength (567nm) exciting the pigment molecules in PS II named as 'light II,' and then frozen to liquid-nitrogen temperature (77K), the fluorescence at 685 nm and 695 nm was repressed and the emission at 715 nm was enhanced (Murata et al., 1966).

To the contrary, illumination with 'light I' at 405 nm plus 435 nm, which was absorbed by PSI, enhanced the emission at 685 nm from PSII (Murata et al., 1966). A regulatory mechanism existed in the algal cells that balanced the distribution of light energy to PSI and PSII depending on the energy of excitation or the quality of light. With an elegant and simultaneous measurement of changes in the oxygen-evolving activity and the fluorescence yield of *Chlorella pyrenoidosa* under 'light I' and 'light II,' Bonaventura and Myers (1969) proposed the concept of - state transitions. This concept is routinely used for decades as 'state 1', referring to photosynthetic organisms exposed to light that is preferentially absorbed by PSI (light I) and 'state 2' to describe photosynthetic organisms exposed to light that is preferentially absorbed by PSII (light II) (Murata, 1970). This phenomena is extended to the energized state of thylakoid membranes. In the presence of ATP, the membranes seemed to establish state 2 and vice-versa. Subsequently, divalent and trivalent ion dependent distribution of light energy between the two photosystems in isolated thylakoid membranes were reported (Murata, 2009).

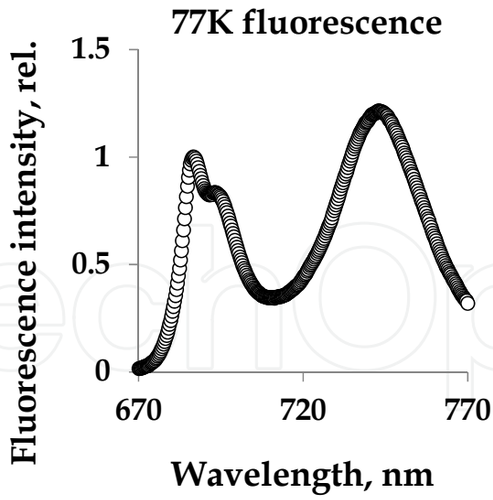
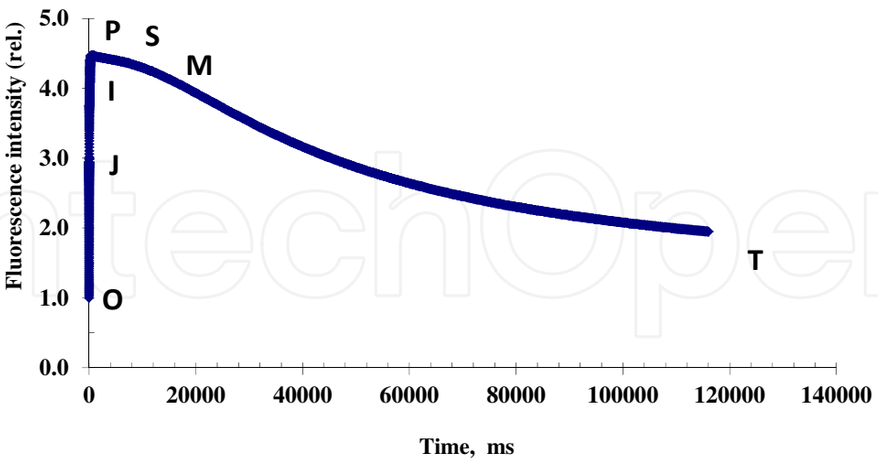


Fig. 5. 77K fluorescence spectrum of a healthy green leaf.

It is now known that under state II light illumination, LHCII becomes phosphorylated by thylakoid membrane localized protein kinase(s), which is regulated by the redox state of the plastoquinone pool (Misra & Biswal, 2000; Zer et al., 2003). The phosphorylated fraction of LHCII then dissociates from PSII and binds to PSI. Reversal of light to state I results in inactivation of the kinase(s), and the LHCII antennae becomes dephosphorylated by constitutively active phosphatases. The (dephosphorylated) LHCII complexes migrate to stacked regions of the grana and re-associate with PSII, restoring its original capacity to absorb light (Allen, 1992; Aro & Ohad, 2003; Mullineaux & Emlyn-Jones, 2005; Rochaix, 2007). This leads to structural changes in the thylakoid membrane itself (Anderson, 1999; Garab & Mustardy, 1999; Dekker & Boekema, 2005). Taking into account of recent developments in several microscopic techniques to study the morphological changes that occur in thylakoid membranes of higher plant chloroplasts during state transitions, Chuartzman et al. (2008) reported that the rearrangements in membrane architecture occurs during the state transition, and involves both granal and stroma lamellar domains. However, due to experimental set-up, repeatability of the experiments and pigment concentration that affects the shape and characteristic of 77K fluorescence, this technique is used to a limited extent and is not as routine as the fast chlorophyll fluorescence or PAM fluorometry as described in the following sections. 77K finds its applications only for conformation of certain temporal and structural orientation of the pigment protein complexes in the thylakoid membranes and energy tunneling within the two photosystems.

2.3 Fast chlorophyll fluorescence

Illumination of dark adapted photosynthetic materials emit, Chl a fluorescence with a characteristic induction or transient which was discovered by Hans Kautsky and is named after him as the Kautsky curve (Kautsky & Hirsh, 1931). Chl a fluorescence induction curve measured under continuous light has a fast (less than a second) exponential phase, and a slow decay phase (few minutes duration). Kautsky curve of a healthy green leaf is shown in Figure 6. The expansion of the fast rise phase gives rise to the exponential 'OJIP' curve (Figure 6). The analysis of the OJIP curve taking the theoretical assumptions and probabilities derives different photosynthetic parameters for the dark adapted state of the



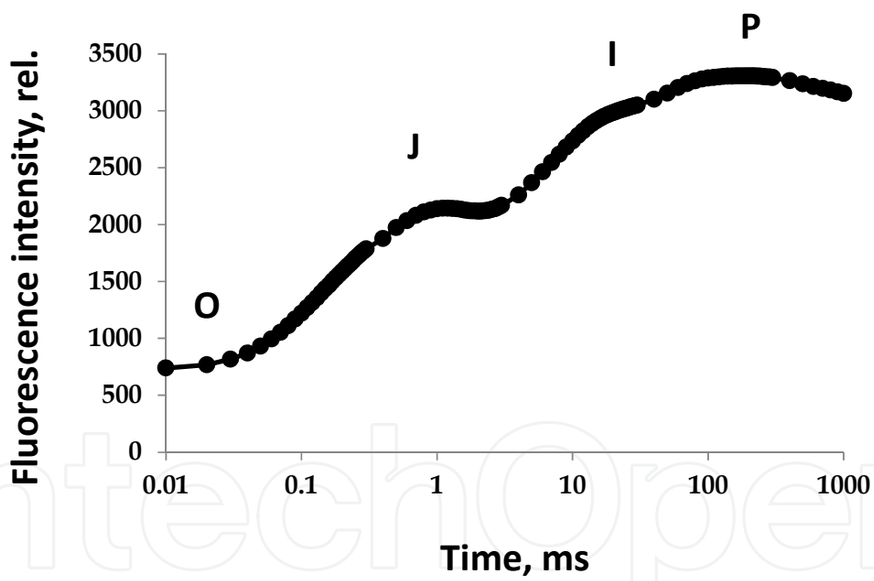


Fig. 6. Kautsky curve of a healthy green leaf and the expansion of the fast rise phase to the exponential 'OJIP' curve. The analysis of the OJIP curve gives rise to different parameters for the dark adapted state of the photosynthetic systems (details in text and also refer Strasser et al., 2004; Stirbet & Govindjee, 2011).

photosynthetic systems (Strasser et al., 2000, 2004; Stirbet & Govindjee, 2011). The slow phase **is** known as 'SMT' and is assigned to a various factors like energy transduction, ATP synthesis, CO_2 fixation, State transition, non-photochemical Chl a fluorescence quenching etc. (Stirbet & Govindjee, 2011). The nomenclature for 'OJIP' is O for origin or F_0 level measured at 20-50µs after illumination, J and I are intermediate states measured after 2ms

and 30 ms, and P is the peak or F_M (maximal fluorescence). In contrast to the angiosperms, the foraminifers, zooxanthellae and lichens, show an additional G peak and H (=P) peak (Tsimilli-Michael et al., 1998; Ilik et al., 2006). The origin G peak is assigned to an early activation of the ferredoxin-NADP+-reductase, FNR, (Ilik et al., 2006). In heat-stressed samples, another peak arises between F_0 and F_J at 300 μs which is designated as K peak (Guisse et al., 1995; Srivastava et al., 1997; Strasser, 1997; Misra et al., 2001b, 2007).

The OJIP curve from F_0 to F_M (=F_P) is correlated with the primary photochemical reactions of PS II (Duysens & Sweers, 1963) and the fluorescence yield is controlled by a PSII acceptor quencher (called ''Q'' = Q_A) (van Gorkom, 1986). Thus, the OJIP transient can be used for the titration of the photochemical quantum yield of PSII photochemistry, and the electron transport properties. As such the OJIP fluorescence curve analysis is routinely used to monitor the effect of various photosynthetic inhibitors, climatic stress, and photosynthetic mutations altering the structure, architecture and function of the photosynthetic apparatus (Misra et al., 2001a, b, 2007; Strasser et al., 2004).

The photosynthetic samples kept in darkness, have the electron acceptor side of PSII in the oxidized state, as there is no electron flow in the photosynthetic electron transport chain and water oxidation by PS II. So the PSII reaction centers remain open, and the fluorescence intensity is minimum, i.e. equal to F_o (='O' level in OJIP curve). On illumination with a strong intensity of light that can theoretically excite all the pigment molecules in the pigment protein bed of the thylakoid membrane, a fast electron transport process takes place and is recorded by a O-J transition or rise within 2 ms. This is followed by slow phases J–I and I–P rise, which are known as thermo sensitive or thermal phases. The F_M level (=P) or F_{max} is attained within 1s, representing a closed PS II centres or complete reduction of all the primary electron acceptor in PS II, the Q_A molecules and saturating the electron flow on the acceptor side of PS II (Schansker et al., 2005). This chapter explains the OJIP curve analysis under saturating light intensities and its use in photosynthetic studies.

The fluorescence induction curve, from photosynthetic samples kept in darkness, are used empirically and commonly using F_0 or F_M values. The difference between F_M and F_0, known as the variable fluorescence, F_V, and the ratio F_V/F_M in a healthy plant ranging from 0.78–0.84 (Bjorkman & Demmig 1987) is used extensively as the maximum quantum yield of primary PSII photochemistry (Butler & Kitajima, 1975; Palliton, 1976). Considering the connectivity parameter or the excitation energy migration among PSIIs (Butler, 1978) and using the relative variable fluorescence at time t, $V_t = (F_t - F_0)/(F_M - F_0)$, the fraction of closed PSII centers (B_t) can be calculated as

$$B_t = [Q_A^-]/ [Q_A^-]_{total}, \text{ since } V_t = B_t/[1 + C (1 - B_t)],$$

where C is probability of connectivity among the PSIIs. When C = 0, or there is no connectivity, $V_t = B_t$. This'separate package model of PSII units' is the fundamental postulate of the JIP test (Strasser et al., 2000; 2004; Tsimilli-Michael & Strasser, 2008). In a recent chapter, Stirbet & Govindjee (2011) revised the JIP-test including the connectivity parameters, as described above, and given a revision of calculations for fluxes and PSII performances as shown in Table 1.

Information selected from the fast OJIP fluorescence induction (data necessary for the calculation of the so-called JIP parameters)	
Fo = F or F	Initial fluorescence after the onset of actinic illumination
F	
F_J F_{2ms}	Fluorescence value at 2 ms (J-level)
F_I F_{30ms}	Fluorescence value at 30 ms (I-level)
F_P(F_M)	Fluorescence maxima under saturating illumination
t_{Fmax}	Time to reach F_M
Area	Area under F_o and F_M
$V_v = F_t - F_o$	Variable Chl fluorescence
$F_V = F_M - F_o$	Maximum variable Chl fluorescence
$V_t = (F_t - F_o)/(F_M - F_o)$	Relative variable Chl fluorescence
$M_o = (dV/d_t)_o = 4$ ms^{-1} . (F - F_o)/(F_V)	Value of the initial slope of curve under V_t
S_m = Area/F_V	Normalized area (proportional to the number of reduction and oxidation of one QA-molecules or the number of electron carriers per electron transport chain)
Energy fluxes (Stirbet & Govindjee, 2011)	
$J^{ABS} = J^{TR} + J^{DI}$	
J^{TR}	Rate of photon absorption by total PSII antenna = *absorbed photon flux*
J_o^{TR}	Rate of exciton trapping (Q_A reduction) by all PSII RCs = *trapped exciton flux*
J_o^{DI}	Maximum (initial) trapped exciton flux
J_o^{ET2}	Rate of energy dissipation in all the PSIIs = *dissipated energy flux*
J_o^{RE1}	Electron transport flux from Q_A to Q_B
	Electron transport flux until PSI acceptors (at F_I level)
Quantum yields and efficiencies	
$_{Po}$ J_o^{TR} / J^{ABS} = 1 F_o/F_M	Maximum quantum yield of primary PSII photochemistry
$_{Pt}$ J^{TR}/J^{ABS} = 1 - F_t/F_M = $_{Po}$ - (1 - V_t)	Quantum yield of primary PSII photochemistry
$_{ETo}$ J_o^{ET2}/J^{ABS} = 1 F_J - F_M = $_{Po}$. (1-V_J)	Quantum yield of the electron transport from Q_A to Q_B
$_{RE1o}$ J_o^{RE1}/J^{ABS} =1 F_1/F_M $_{Po}$. (1-V_I)	Quantum yield of the electron transport upto the PSI electron acceptors
$_{ET2o}$ J_o^{ET2}/J_o^{TR} = 1 - V_J	Efficiency of trapped electron transfer from Q_A to Q_B
$_{ET1o}$ J_o^{RE1}/J_o^{TR} = 1 V_I	Efficiency of electron transfer from PSII to PSI acceptors
$_{RE1o}$ J_o^{RE1}/J_o^{ET2} = (1 V_I)/ (1-V_j)	Efficiency of electron transfer from Q_B to PSI acceptors
Specific energy fluxes (per active PSII RC)	
$J^{ABS}S/RC = (M_o/V_J)$. (1/ $_{Po}$)	Absorbed photon flux per PSII RC (apparent antenna size of active PSII)
$_{RC2} = Chl_{RC}/Chl_{tot}$	Probability that a PSII Chl functions as RC
RC/J^{ABS} = $_{Po}$. V_J/M_o = $_{RC2}$ /(1 - $_{RC2}$)	Number of Q_A reducing RCs per PSII antenna Chl
J_o^{TR} /RC = M_o/V_J	Maximum trapped exciton flux per PSII
J_o^{ET2}/ RC = (M_o/V_J) . (1 - V_J)	Electron transport from Q_A to Q_B per PSII RC
J_o^{RE1}/RC =(M_o/V_J) . (1 V_I)	Electron transport to PSI acceptors per PSII RC
Phenomenological energy fluxes (per CS_o)	
$J^{ABS}/CS_o = F_o$ or $J^{ABS}/CS_M = F_M$	Absorbed photon flux per cross section (apparent PSII antenna size)
RC/CS = (RC/J^{ABS}) . (J^{ABS}/CS)	The number of active PSII RCs per cross section
J_o^{TR} /CS = (J_o^{TR} / J^{ABS}) . (J^{ABS}/ CS)	Maximum trapped exciton flux per cross section
J_o^{ET2}/CS = (J_o^{ET2}/J^{ABS}) . (J^{ABS}/ CS)	Electron transport from Q_A to Q_B per cross section
J_o^{RE1}/CS = (J_o^{RE1}/J^{ABS}) . (J^{ABS}/ CS)	Electron transport flux until PSI acceptors per cross section
De-excitation rate constants of PSII antenna	
$k_N = k_F - J^{ABS}/F_M$	Non-photochemical de-excitation rate constant; k_F = rate constant of fluorescence emission
$k_P = k_F - J^{ABS} - F_V/(F_o - F_M) = k_N - F_V/F_o$	Photochemical de-excitation rate constant
Performance index	
PI_{ABS} =[$_{RC2}$/(1 - $_{RC2}$)].[$_{Po}$/(1- $_{Po}$)].[$_{ET2o}$/(1- $_{ET2o}$)]	Performance index for energy conservation from photons absorbed
$PI_{ABS}^{tota} = PI_{ABS}$. [$_{RE1o}$/(1- $_{RE1o}$)]	

Table 1. Equations and definitions of JIP parameters by Strasser et al. (2004; 2010) and modified by Stirbet & Govindjee (2011)

2.4 PAM fluorescence

The widely used chlorophyll fluorescence technique is the so-called quenching analysis of modulated fluorescence by the saturation pulse method. In this type of measurement system instead of using a continuous light, a high intensity light mimicking the 'sun light intensity' is switched on and off (pulse) at high frequency and the detector is tuned to measure the fluorescence emission only, thereby providing a more efficient and more powerful system to measure fluorescence emission in presence of background measuring light (Bradbury & Baker, 1981; Quick & Horton, 1984; Schreiber et al., 1986; Schreiber, 2004). A leaf is dark adapted for at least 10-15 min prior to the measurement. The ground fluorescence (F_o) in darkness is measured by a weak modulating light beam (ML). Then the application of a saturating pulse (SP) (about 8000 μmol m^{-2} s^{-1} for 0.6 - 1 s), raises the fluorescence to a maximum value, F_m. This measurement allows the determination of the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given as $\underline{F_v/F_m}$, as described for the fast chlorophyll fluorescence measurements described in earlier section. This parameter is often called as 'intrinsic quantum yield' (Kitajima & Butler, 1975). Initially after this first light pulse the actinic light (AL) is switched on (photosynthetic samples are illuminated) and SP is turned on repeatedly. This induced F_m' (fluorescence maxima at light adapted state). The F_m' increases initially with few pulses and then starts declining (quenching) after few minutes. The intial phase of rise in fluorescence in light adapted state is called 'photochemical quenching' which is ascribed to the photochemical phenomena in generating reductants and subsequent reduction of carbon dioxide pool in the leaves (van Kooten & Snell, 1990; Edwards & Baker, 1993) subsequent pulses of saturating light interrupted with dark period gradually reduces the intensity of fluorescence emission otherwise known as 'non-photochemical fluorescence quenching' or NPQ (Walter & Horton, 1991; Johnson et al., 1993; Oxbrough & Baker, 1997, Niyogi et al., 1997). A typical PAM fluorescence measurement is shown in Figure 7. The calculation of quenching parameters

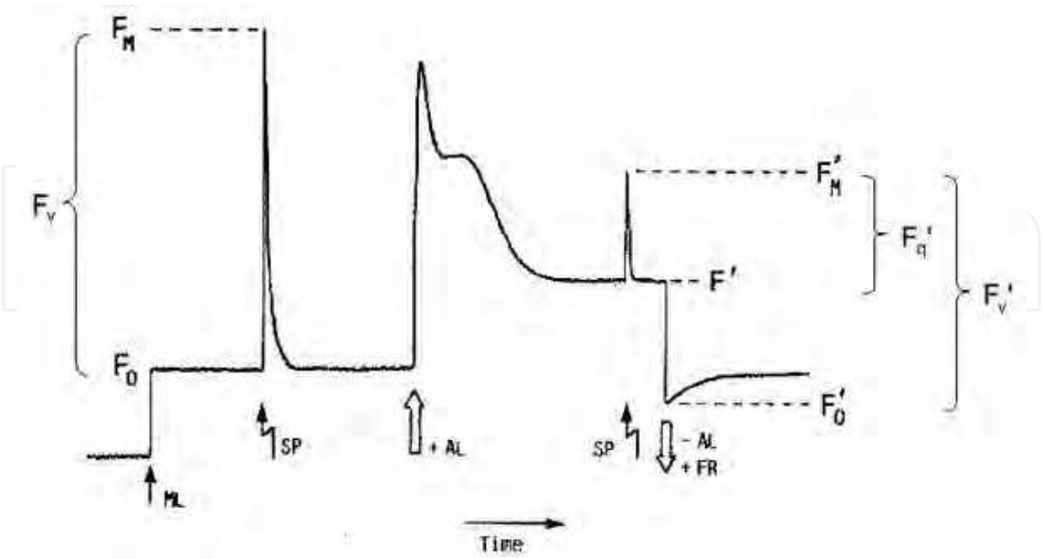


Fig. 7. A typical PAM fluorescence signal of a leaf disc. The fluorescence in dark adapted leaves are denoted by F and in the light adapted state F' are recorded and different quenching parameters are measured (see Text).

needs either a shift from NPQ to photochemical quenching or vice versa. It is not practicable to shift completely away from NPQ to a complete photochemical quenching situation, so the alternative of complete shift from photochemical quenching to a NPQ stage is suggested by many workers (Bradbury & Baker, 1981; Quick & Horton, 1984). The terminology suggested by van Kooten & Snell (1990) and then modified by Maxwell & Johnson (2000) and Baker (2008) is used widely.

The sample is first dark adapted. The test is started and F_o, or minimal fluorescence, is measured without actinic light. Then a saturation pulse (SP) completely closes all the primary electron acceptors (Q_A) in PSII by completely reducing PSII. So maximal fluorescence, F_m, is the result. After the saturation pulse, an actinic light is turned on and the fluorescent signal declines slowly with the onset of CO_2 fixation until it reaches steady state. Photochemical quenching a measure of open PSII centers, photo-protective non-photochemical quenching and other heat dissipation mechanisms occur. Saturation pulses during steady state photosynthesis provide F_m', maximal fluorescence in light adapted state, after NPQ has reached equilibrium with photochemistry. qP, or qL, now represents the fraction of PSII receptors that remain open or oxidized. F′ (or F_s) represents fluorescence related to current steady state photochemical levels. Then the actinic light is turned off, and simultaneously far red (FR) illumination is turned on to allow the transfer of electrons quickly to reduce PSI, and allow the re-oxidation of PSII. F_o' represents this value with un-relaxed non-photochemical quenching. The rising values of the saturation pulses after the actinic light has been turned off represent the relaxation of NPQ over time. A portion of NPQ, qE (or Y(NPQ), represents photo-protection mechanisms of thylakoid lumen ΔpH and the xanthophyll cycle. The remainder of NPQ represents qT, and qI, (or Y(NO). qT is quenching due to state 1 and state 2 transitions and is negligible in higher plants. qI represent photo-inhibition and photo-damage (adapted from Fracheboud & Leipner 2003; http://www.ab.ipw.agrl.ethz.ch/ ~yfracheb/ flex.htm).

2.4.1 Photochemical quenching

As shown in the fast Chl fluorescence measurement, the maximum quantum efficiency of PSII photochemistry is calculated as:

$$F_v/F_m = (F_m\text{-}F_o) \,/\, F_m$$

A decrease in F_m and/or an increase in F_o results in a decrease in F_v/F_m. The F_o increase is provoked by dissociation of LHCII from the PSII core complex and is reported to be due to the free pigments (Misra & Terashima, 2003; Misra et al., 2001a,b, 1998, 2007).

In natural conditions, sun light far exceeds the quantum requirements for photochemistry in photosynthesis, commonly referred as 'photoinhibition' (Misra, 1993; Misra et al. 1997; 2001; 2007). Under these conditions, the PSII RC undergoes photoinduced damages of the D1 protein. The first turn-over of this polypeptide copes up with the photoinhibitory situations. However, under severe stress, the capacity for repair of damaged PSII RC becomes suboptimal and an irreversible inhibition of PSII can be detected *in vivo* as a decrease in the chlorophyll fluorescence ratio F_v/F_m. So F_v/F_m is often used as a useful parameter to estimate the extent of photoinhibition of photosynthesis. However, when NPQ induces a decrease in F_v/F_m, this quantification can be erroneous. However, under photoinhibitory conditions, NPQ is lowered due to low F_m signal (Misra et al., 2006, 2011). Since

photoinhibition will reduce the excitation pressure on the reducing site of PSI, these leaves are often characterised by higher values of F_q'/F_v' (Misra et al. 2003, 2006, 2011).

The application of a SP in the presence of AL allows the determination of the maximum fluorescence in the light-adapted state (F_m') or of the PSII 'open centres'. But F_m' shows a decrease compared to that of F_m value, indicating the presence of NPQ. Genty et al. (1989) proposed the 'photochemical quenching' which later became popularly known as 'Genty parameter' and is calculated as:

$$F_q'/F_m' = (F_m'-F') / F_m'$$

Theoretically 'Genty parameter' is proportional to the quantum efficiency of PSII photochemistry in the light adapted state (PSII quantum efficiency = Φ_{PSII}), which is affected by the level of electron acceptors, e.g. $NADP^+$, available at the acceptor side of PSI (Oxborough & Baker, 1997). However, F_q'/F_m' is greatly affected by the light intensity. So precaution has to be done during measurements under natural conditions where changes in the incident sun light intensity is frequented. This terminology is also used in the literature as Φ_{PSII}, $\Delta F/F_m'$, $(F_m'-F_t)/F_m'$ and $(F_m'-F_s)/F_m'$ (where $\Delta F = F_q'$, and F_t and F_s is equal to F'). Both, the changes in the electron flux on the reducing side of PSII and the down-regulation of PSII affects F_q'/F_m', as this is the product of $\underline{F_q'/F_v'}$ (PSII quantum efficiency factor = coefficient of photochemical quenching (qP) and $\underline{F_v'/F_m'}$ (maximum quantum efficiency of PSII). $\underline{F_v'/F_m'}$ is affected by antenna quenching. F_q'/F_v' or qP is an approximation of the redox state of the primary electron acceptor Q_A in the light adapted state.

qP is a measure of the fraction of open PSII reaction centers and is defined as the coefficients of photochemical fluorescence quenching (van Kooten & Snel, 1990). In cases where qN is greater than 0.4 this may not be a good assumption. Under such a condition, the calculation of qN and qP values are affected. So another parameter – Fod is introduced to minimize the effect of qN on the calculation of qP (van Kooten & Snel, 1990). Kramer et al. (2004) used qL as photochemical quenching parameter. It is a measure of the fraction of open PSII reaction centers. 1- qP, reflects the proportion of closed centers or the "excitation pressure" on PS II (Maxwell et al.,1994; Misra et al., 2006, 2011).

2.4.2 The rate of linear electron transport in PSII (ETR)

The electron transport rate in PSII (ETR) can be calculated as proposed by Fryer et al. (1998):

$$\text{ETR} = F_q'/F_m' \cdot \text{PFD} \cdot a_L \cdot (\text{PSII}/\text{PSI})$$

Where:
PFD is the photosynthetic photon flux density in µmol quanta m^{-2} s^{-1}, measured with a quantometer;
a_L the leaf absorbance, measured with an integrating sphere; and
PSII/PSI = proportion of light absorption by PSII and PSI (assumed value).

The maximum ETR is the sum total of all electron sinks in a chloroplast such as carbon fixation, photorespiration, nitrate assimilation, Mehler reaction. A perturbation or change in any of these parameters affects ETR.

2.4.3 Non-photochemical quenching (NPQ)

Non-photochemical quenching of chlorophyll fluorescence is an indicative of the level of non-radiative energy dissipation in the LHC II of PSII, which is ascribed to prevent over-reduction of the electron transfer chain and, therefore, provides protection from photodamage. The parameter NPQ is derived from the Stern-Volmer equation and can be used to follow changes in apparent quencher concentration (Bilger & Bjorkman, 1990). *NPQ* is related to the rate constant for excitation quenching by regulated thermal dissipation (k'_N). Non photochemical quenching is measured in plants by several methods depending on the NPQ limitations. NPQ – the non-photochemical quenching is a measure of heat dissipation and is the sum total for the photo-protective mechanisms, state transition quenching, and photo-inhibition (Krause and Weis, 1991; Muller et al., 2001; Finazzi et al., 2006).

$$NPQ = qE + qT + qI.$$

NPQ is calculated as: $NPQ = (F_m/F_m') - 1$

NPQ can occur even at low light intensity. Stress conditions such as high light intensity or photoinhibition, low internal CO_2 concentration due to drought or chilling (low temperature) accelerate NPQ. So NPQ serves as an index of stress. At moderate light intensity, the NPQ steady state value is temperature dependent. However, NPQ saturates after a specific temperature limiting the capacity of quencher, which is altered by acclimation. Low temperature decreases the rate of NPQ development irrespective of the light intensity. Bilger and Björkman (1991) demonstrated that the development of NPQ upon exposure of leaves to excess light is, at least partially, determined by the rate of zeaxanthin formation (Misra et al., 2006, 2011). In higher plants, NPQ is divided into two different components (i) rapidly relaxing ΔpH- or energy-dependent NPQ, known as q_E and (ii) a slower photoinhibitory NPQ, known as q_I. qE is ΔpH dependent and depends on the xanthophyll cycle dependent photo-protective mechanisms in the leaf, qT value is negligible in higher plants and so increasing value of q_I indicates enhanced stress in higher plants (Muller et al. 2001).

This is independent of F_o estimation or the quantification of 'closed' PSII RCs and reflects heat-dissipation of 'excess excitation energy' in the antenna system.

qN is similar to NPQ but requires Fod (dark adapted state after a far-red illumination) or F_o' (light adapted state) for estimation. qN is defined as the coefficient of non-photochemical fluorescence quenching. The assumptions for using qN is that it affects primarily the 'variable fluorescence' (F_v) and not the F_o and qN is not greater than 0.4. By using the Far-Red source after actinic illumination, the PSII acceptors re-oxidized and PSI is reduced. A new Fod value is measured and used for corrections to the quenching coefficients (van Kooten & Snel, 1990). NPQ is relatively insensitive to the part of non-photochemical quenching associated with qN values lower than 0.6 This range of qN is affected by ΔPH of the thylakoid lumen which is an important aspect of photosynthetic regulation. (Bilger & Björkman, 1990). Kramer et al. (2004) introduced new quenching parameters such as Y(NPQ) that represents heat dissipation related to all photo-protective mechanisms and Y(NO) represents all other components that are not photo-protective.

2.4.4 Calculations for quenching parameters

$qP = (F_m' - F) / (F_m' - F_o')$
$NPQ = (F_m - F_m') / (F_m')$
$NPQ = qE + qT + qI$
$qE = F_m'$ after rapid relaxation is complete with the actinic light turned off usually one to ten minutes - F_m' during steady state fluorescence with actinic light on/F_m' at steady state.
$qT = F_m'$ after rapid relaxation is complete usually with the actinic light turned off usually one hour - F_m' at qE /F_m' at steady state.
$qI = F_m - F_m'$ at qT/ F_m' at steady state.
$qN = F_m - F_m' / F_m - F_o$
$qL = qP(F_o'/F')$
$Y(NO) = 1/NPQ + 1 + qL((F_m/F_o)-1)$
$Y(NPQ) = 1 - Y - Y(NO)$
$1 = qL + Y(NPQ) + Y(NO)$

3. Applications of chlorophyll fluorescence measurements in plant biology

The primary use of fluorescence has been the estimation of chlorophyll concentration and pigment-protein interaction studies, stability of thylakoid membranes etc. However, the relationship between chlorophyll and in vivo fluorescence varies with a wide range of time and space. These processes included species changes, nutrient concentrations, incident radiation, etc (Falkowski & Raven, 2007). The use of sun-stimulated fluorescence to estimate primary productivity is suggested.

Not only that the fluorimetric techniques are used for aquatic plant productivity, but also these chlorophyll fluorescence measurements, have a wide range application in the field of forestry, crop or plant productivity estimates and in stress adaptation studies (for reviews see Sayed, 2003; Baker & Rosenquivst, 2004; Rohacek et al., 2008; Strasser et al., 2004; Tsimilli-Michael et al., 1998; Tsimilli-Michael & Strasser,2008; Srivastava et al., 1995, 1997).

An extensive study is done on the application of fluorimetry especially PAM and fast Chl fluorimetry on the stress adaptation studies in plants. The most widely studied stress is 'photoinhibition' as this process is related to the fundamental principle of fluorescence energy quenching. The role of the xanthophyll cycle in non-photochemical quenching is the most interesting out come of these photoinhibitory studies using fluorescence parameters (Demmig-Adams, et al., 1996; Frank et al., 1994; Horton et al., 1994; Misra et al, 2003; 2006; 2011).

Recently, chlorophyll fluorescence is used as one of the sensitive parameters for biosensors using thylakoid membranes or algal cells as the transducers (Apostolova et al., 2011; Dobrikova et al. 2009; Giardi & Pace, 2005; Koblizek et al., 1998; Misra et al., 2003, 2006, 2011; Raskov et al., 2011; Vladkova et al, 2009, 2011). Besides this fast Chl fluorescence can be used as a sensitive device for detection of ion/ salt sensitivity and other environmental stress factors (Misra et al., 2001a,b, 2007).

A consorted effort on the improvement of the instrumentation, miniaturization and quickness of the data acquisition will help in further information flux in this field which still has a wide scope and utility.

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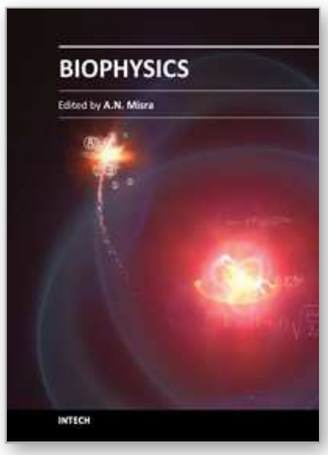

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Biophysics is a vast cross-disciplinary subject encompassing the fields of biology, physics and computational biology etc in microbes, plants, animals and human being. Wide array of subjects from molecular, physiological and structural are covered in this book. Most of these chapters are oriented toward new techniques or the application of techniques in the novel fields. The contributions from scientists and experts from different continents and countries focuss on major aspects of biophysics. The book covers a wide range of topics reflecting the complexity of the biological systems. Although the field of biophysics is ever emerging and innovative, the recent topics covered in this book are contemporary and application-oriented in the field of biology, agriculture, and medicine. This book contains mainly reviews of photobiology, molecular motors, medical biophysics such as micotools and hoemodynamic theory.

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