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A comparison of chlorophyll fluorescence transient measurements, using *Handy PEA* and *FluorPen* fluorometers

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Abstract

We provide here a general introduction on chlorophyll (Chl) *a* fluorescence, then we present our measurements on fast (< 1 s) induction curves (the so-called OJIP transients) on dark-adapted intact leaves of *Arabidopsis thaliana*, under five different light intensities [in the range of ~ 500 to ~ 3,000 µmol(photons) m^{-2} s^{-1}] using two different instruments: *Handy PEA* (*Hansatech Instruments*, UK; excitation light, 650 nm) and *FluorPen* (model *FP-110*; *Photon Systems Instruments*, The Czech Republic; excitation light, 470 nm). We then discuss the observed differences in the OJIP curves, as well as in F_o ($F_{20\mu s}$, $F_{50\mu s}$, or the extrapolated $F_{t\to0}$), F_P (the peak), and the ratios F_P/F_o, and F_v (= $F_P - F_o$)/F_P in terms of differences in excitation light intensity and absorptance (or absorbance) of the excitation light by the leaves, and other factors, as well as the data available in the literature. We suggest that such measurements be accompanied, in the future, by parallel measurements on Chl *a* fluorescence imaging, an area pioneered by Hartmut K. Lichtenthaler.

Keywords: *Arabidopsis thaliana*; blue and red excitation light; fluorescence imaging; OJIP transient.

Introduction

Light energy absorbed by cyanobacteria, algae, and plants has three main fates: photochemistry (the process which drives photosynthesis), heat dissipation in the antenna of the photosystems, and chlorophyll (Chl) *a* fluorescence. Although Chl *a* fluorescence is only 2–3% of the total absorbed light (Duysens 1979, Trissl *et al.* 1993), its

Highlights

- OJIP Chl *a* transient from *Arabidopsis* was measured with two commercial fluorometers
- F_o, F_P, and F_v/F_P values were obtained with blue *vs.* red light, at five light intensities
- We suggest parallel measurements with fluorescence imaging and lifetime of fluorescence

Abbreviations: Chl – chlorophyll; F_m – maximum Chl *a* fluorescence; F_o – initial (minimal) Chl *a* fluorescence; F_v (= $F_m - F_o$) – variable Chl *a* fluorescence; I-step – Chl *a* fluorescence at ~ 30 ms; J-step – Chl *a* fluorescence at ~ 2 ms; OJIP curve – the 'fast' phase of the fluorescence transient ['O' is for the initial fluorescence (at ~ zero time), 'P' is for peak, and 'J' and 'I' are inflection points between 'O' and 'P']; PQ – plastoquinone; Q_A and Q_B – the first and second plastoquinone electron acceptor of PSII.

Conflict of interest: The authors declare that they have no conflict of interest.

measurement provides insight into the photosystems as well as the overall process of photosynthesis (Govindjee et al. 1986, Shevela et al. 2019, Blankenship 2021). Light absorption by Chl a molecules leads to the formation of excited states (within femtoseconds), which then decay to ground state by losing energy through photochemistry (k_p), internal conversion (k_{IC}), and fluorescence (k_f); these are picosecond (ps) to nanosecond (ns) processes; they are competitive in nature, i.e., change in the probability of one leads to changes in probabilities of the other two. Hence, by measuring Chl a fluorescence yield, we can gain knowledge about photochemistry and heat dissipation (see e.g., Govindjee 2004, Jahns and Holzwarth 2012, Ruban et al. 2012, Murchie and Lawson 2013, Lazár 2015; also chapters in Demmig-Adams et al. 2014).

A popular method, to characterize photosynthetic samples, has been to use light intensity-dependent changes in Chl a fluorescence emission of dark-adapted photosynthetic samples, during few minutes of illumination with continuous light (Kautsky and Hirsch 1931, Govindjee 1995), known as Chl a fluorescence induction or fluorescence transient; during this time, electron transport in both PSI and PSII occur, followed by the Calvin-Benson cycle (Krause and Weis 1991, Maxwell and Johnson 2000, Baker 2008).

Chl a fluorescence induction has been an indispensable tool, for a long time, in the study of various aspects of photosynthesis (see e.g., Neubauer and Schreiber 1987, Schreiber and Neubauer 1987, Lichtenthaler 1988, Lazár 1999, Schansker et al. 2005, 2014; Kalaji et al. 2014, 2017; Stirbet et al. 2020), including the structure, as well as the function of the photosynthetic systems (e.g., Kaňa et al. 2012, Lazár 2013, Stirbet et al. 2019, Schreiber and Klughammer 2021; chapters in Papageorgiou and Govindjee 2004). The initial part of the above-mentioned fluorescence transient curve, up to hundreds of milliseconds, has been labeled in the literature as the 'fast' phase of the fluorescence transient (also known as the OJIP transient), while the rest of the curve up to minutes, as the 'slow' phase of the fluorescence transient (see e.g., Papageorgiou and Govindjee 1968, Govindjee and Papageorgiou 1971, Bradbury and Baker 1981, Govindjee 1995, Papageorgiou et al. 2007, Stirbet and Govindjee 2011, 2016). Since these measurements are noninvasive, they are useful for obtaining a better understanding of the photochemical (and subsequent electron transfer) reactions of cyanobacteria, algae, as well as plants; further, this method is also used under field conditions, since the available commercial fluorometers are quite compact, and thus portable. Analysis of Chl a fluorescence induction in plants provides us with information on both PSI and PSII, especially on the maximum quantum yield of PSII photochemistry, as well as on the photosynthetic electron transport and different nonphotochemical quenching (NPQ) mechanisms in the system (see e.g., Genty et al. 1989, Kramer et al. 2004, Schreiber 2004, Strasser et al. 2004, Schreiber and Klughammer 2007, Stirbet and Govindjee 2011, Stirbet et al. 2018).

In fluorometers, used to measure Chl a fluorescence induction, different methods are utilized to saturate photochemistry (Röttgers 2007); further, the wavelength of the excitation light (Schreiber et al. 2012), as well as the detection window for fluorescence emission, are often different. We note that direct fluorometry with LED-based shutterless instruments (saturating pulse measurement) and pulse amplitude modulation (PAM) fluorometry (steady-state measurement; Schreiber 2004) are the ones that are most commonly used (see e.g., Kalaji et al. 2014). The color of the excitation light is important, as well as the detection window since the two photosystems (I and II) have distinct absorption and emission characteristics (see e.g., Evans 1986). Excitation light in the red, as well as in the blue regions of the spectrum is usually used in the measurement of fluorescence induction in plants. Since there are differences between the effects of red and blue light with the leaves (Vogelmann and Han 2000), we decided to study the fast (< 1 s) Chl a fluorescence, from the 'O' level to the 'P' level (the OJIP transient), using these two lights, on leaves of Arabidopsis thaliana, a model plant. We also recorded the F_v ($= F_m - F_o)/F_m$ ratio, a proxy for the quantum yield of PSII photochemistry, which is also used as a sensitive indicator of plant photosynthetic performance (see e.g., Stirbet et al. 2018). For this work, we used two commercially available instruments: Handy PEA (Plant Efficiency Analyzer, Hansatech Instruments, UK; excitation $\lambda = 650$ nm) and FluorPen FP-110 (Photon Systems Instruments, The Czech Republic; excitation $\lambda = 470$ nm).

Background and meaning of the OJIP transient

By plotting Chl a fluorescence changes (measured on dark-adapted samples) on a logarithmic time scale up to ~ 1 s (see e.g., Fig. 1), one observes a distinct polyphasic induction curve, the so-called OJIP transient (Strasser and Govindjee 1992, Strasser et al. 1995). The fast Chl fluorescence rise, starting from the 'O' level (the minimum fluorescence F_o, usually approximated by fluorescence at 20 or 50 μs of illumination), increases to a peak 'P' with two intermediary steps, 'J' (fluorescence at 2 ms, F_J) and 'I' (fluorescence at 30 ms, F_I). The F_o is also referred to as the initial fluorescence, or 'origin'; it is due to energy loss in the antenna pigments before the excitation energy is trapped at the reaction centers, when all PSIIs are 'open' [i.e., with Q_A, the first plastoquinone (electron) acceptor of PSII, in the oxidized state].

The reduction of Q_A to Q_A^-, as well as that of Q_B to Q_B^- (by Q_A^-), predominate during the OJ phase (the photochemical phase), while at the end of the JI phase, the PQ pool is greatly ($\geq 80\%$) reduced (see e.g., Lazár 2009). Further, during the IP phase, since both the photosystems are simultaneously excited, the PQ pool, the Cyt b_6f complex and plastocyanin (PC, which reduces P700$^+$, the oxidized primary donor of PSI), and the electron carriers beyond P700, up to ferredoxin (Fd), are all reduced during this phase. [We are aware that ferredoxin–NADP$^+$-reductase in plants remains inactive for a few seconds after the onset of illumination, until the stroma pH increases to a value ~ 8, when it is activated (Pschorn et al. 1988).] When the OJIP rise is measured using saturating light, the P level has the maximum value (F_m), as the electron transport chain becomes completely reduced. The ratio

between the variable fluorescence ($F_v = F_m - F_o$) and F_m (*i.e.*, F_v/F_m), which is a proxy of the maximum quantum yield of PSII photochemistry (Kitajima and Butler 1975, Genty *et al.* 1989), is one of the most used parameters obtained from the OJIP transients. Also, another well-known parameter is the F_v/F_o ratio, which is equivalent to k_P/k_N (*see e.g.*, Tsimilli-Michael 2020), where k_P is the photochemical de-excitation rate constant, and k_N is the nonphotochemical de-excitation rate constant of PSII (*see* Papageorgiou and Govindjee 2011). We recommend that, to obtain a complete picture, parallel measurements with Chl *a* fluorescence imaging systems be included with measurements, described above (*see e.g.*, Lichtenthaler *et al.* 1996, Buschmann and Lichtenthaler 1998).

Materials and methods

In this study, we used four-week-old wild type *Arabidopsis thaliana* plants, grown in agro peat/vermiculite (3:1) mixture in pots, under illumination with cool white, fluorescent light [100 μmol(photons) m^{-2} s^{-1}], with 10-h L/14-h D photoperiod. The temperature in the room was maintained at $21 \pm 1°C$, and the plants were watered twice a week. Essentially, almost identical bottom leaves were used during the measurements made with the two fluorometers (*see* below), since different leaves from the same plant are known to have differences in their fluorescence transients (*see e.g.*, Küpper *et al.* 2019).

The OJIP transients were measured with both *Handy PEA* and *FluorPen FP-110* instruments. The 'emitter' for *Handy PEA*, a LED array, is centered at 650 nm with NIR short-pass filters, whereas its detector is a photosensor with *Kopp Corning* RG-long-pass filter, for wavelengths longer than 700 nm. In contrast, *FluorPen FP-110* is equipped with a LED (*OPTOSUPPLY OSB56L5111Y*) centered at ~ 470 nm, and its detector is a PIN photodiode with bandpass filters in the 667–750 nm range. The maximum (100%) intensity of light, from both the instruments (as given in their respective manuals) is 3,000 μmol(photons) m^{-2} s^{-1}. However, we measured the intensity of the excitation light provided by each of the two fluorometers, by using a *LI-COR* Quantum Radiometer (model *LI-189*)

by placing the light sensor in the leaf holder of both the instruments (*see* the results in Table 1); on the average, the maximum intensity of the excitation light in *FluorPen* had ~ 10% higher intensity than in the *Handy PEA* (where relevant, we will take this into account when comparing data by the two instruments). This instrument (as well as *LI-190R*) measures PAR over the 400–700 nm range, which provides energy, but then it is converted by its program, into the appropriate number of photons (light quanta). The sensitivity is automatically set, in the instrument, in such a way, that it takes into account the fact that blue photons (quanta) have more energy than the red quanta; thus, it correctly reads the quantum flux rates [μmol(photons) m^{-2} s^{-1}] of the blue and red light.

Before measurement, intact leaves of *Arabidopsis thaliana* were dark-adapted for 20 min using leaf clips, then Chl *a* fluorescence transients (of 1-s duration, at room temperature) were measured with each instrument, under five different incident light intensities of 100, ~ 80, ~ 50, ~ 30, and ~ 20% [where 100% light intensity was $2,890 \pm 70$ and $3,120 \pm 87$ μmol(photons) m^{-2} s^{-1} for *Handy PEA* and *FluorPen*, respectively; *see* Table 1]. The OJIP transients were analyzed with the software provided by *Photon Systems Instruments* (for *FluorPen*) and by *Hansatech Instruments* (for *Handy PEA*). The fluorescence parameters used in this work included (*1*) the initial fluorescence F_o which was estimated either as the fluorescence measured at 20 μs ($F_o = F_{20μs}$), or at 50 μs ($F_o = F_{50μs}$), or extrapolated to t = 0 ($F_o = F_{t→0}$), calculated by fitting the first several fluorescence points (in the range of 20 to 80 μs) of the transient with a line that intersected the ordinate at t = 0; (*2*) the fluorescence at the peak P, F_P; (*3*) the variable fluorescence F_v (= $F_P - F_o$); and (*4*) the F_v/F_P ratio. (Note that for *Handy PEA*, *Hansatech* uses $F_{20μs}$ as an approximation for F_o, and for *FluorPen*, *Photon Systems* uses $F_{50μs}$ for the same.)

Results

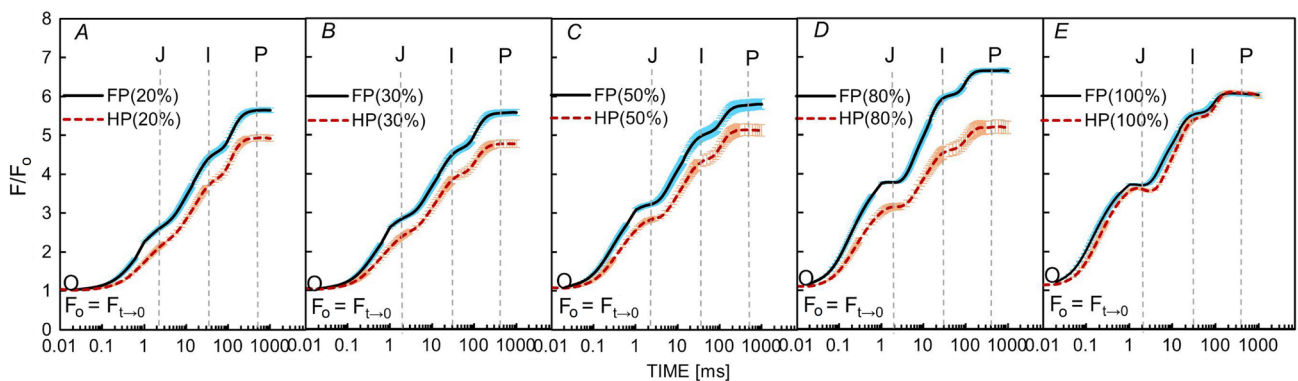Fig. 1 shows the OJIP transients measured with FluorPen and Handy PEA instruments under 100%, and then

Fig. 1. The OJIP, Chl *a* fluorescence induction, curves normalized to the extrapolated $F_{t→0}$ as F_o (*see* 'Materials and methods'), as measured with *Handy PEA* (HP; excitation light, λ = 650 nm) and *FluorPen* (FP; excitation light, λ = 470 nm) on 20-min dark-adapted intact leaves of wild type *Arabidopsis thaliana*, under 100% and lower light intensities [where 100% light intensity is $2,890 \pm 70$ and $3,120 \pm 87$ μmol(photons) m^{-2} s^{-1} for HP and FP, respectively; *see* Table 1]. The curves, shown here, are averages of 15 replicates ± SE.

approximately 80, 50, 30, and 20% excitation light intensity (for details, *see* 'Materials and methods'); for comparison, Chl a induction curves were normalized to F_o extrapolated at t = 0 ($F_{t\to0}$). We observed here clear differences between the OJIP curves obtained by the two instruments. For example, the O–J phase (which is dependent on how much light is absorbed by the antenna pigments of PSII) rose slower and was smaller in the samples measured with *Handy PEA* (using red light) than those measured with *FluorPen* (using blue light); this is partly due to approximately 10% higher excitation light (*see* above), and to higher absorptance (absorption) of blue light compared to red light (*see e.g.*, McClain and Sharkey 2020). Further, the F_P/F_o ratio was higher for *FluorPen* than *Handy PEA* at almost all the light intensities used (*see* Fig. 1). At 100% light, F_P/F_o was essentially the same with both the instruments (Fig. 1E); the value of F_P/F_o with *FluorPen* was lower here due to a higher increase in $F_o = F_t\to0$ (Table 2). However, at 80% light, the F_P/F_o with *FluorPen* was the highest (Fig. 1D) as $F_o = F_t\to0$ was

Table 1. A comparison of measured light intensities [µmol(photons) m^{-2} s^{-1}], from *Handy PEA* and *FluorPen* fluorometers (*right columns*), with the published values in the manuals (*left column*). Instrument used: *LI-COR* Quantum Radiometer, model *LI-189* (also *see* 'Materials and methods'). Data are averages of three measurements ± SE.

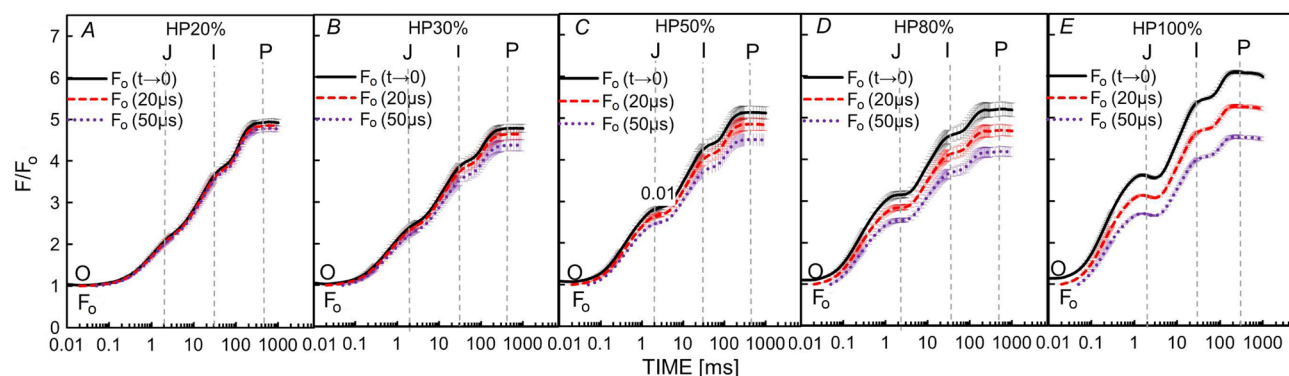| Light intensities, from instrument manuals	Measured light intensities (for *Handy PEA*)	Measured light intensities (for *FluorPen*)
3,000 (100%)	2,890 ± 70	3,120 ± 87
2,400 (80%)	2,314 ± 52	2,380 ± 57
1,500 (50%)	1,496 ± 37	1,534 ± 55
900 (30%)	832 ± 39	923 ± 38
600 (20%)	515 ± 36	598 ± 45

Fig. 2. Chl *a* fluorescence induction (OJIP) curves normalized to F_o extrapolated at t = 0 ($F_{t\to o}$), $F_{20\mu s}$, and $F_{50\mu s}$, as measured with *Handy PEA* (excitation light, λ = 650 nm) on 20-min dark-adapted intact leaves of wild type *Arabidopsis thaliana*, under different light intensities [where 100% light intensity is 2,890 ± 70 µmol(photons) m^{-2} s^{-1}; *see* Table 1]. The curves are averages of 15 replicates ± SE.
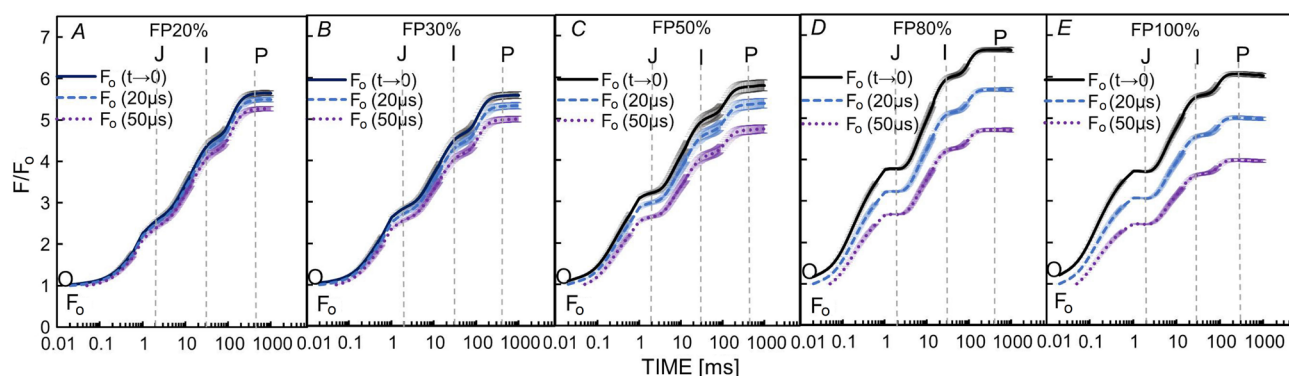
Fig. 3. Chl *a* fluorescence induction (OJIP) curves measured under different light intensities and normalized to F_o (extrapolated) at t = 0 ($F_{t\to o}$), $F_{20\mu s}$, and $F_{50\mu s}$. Data obtained with *FluorPen* (FP) (excitation light, λ = 470 nm) on 20-min dark-adapted intact leaves of wild type *Arabidopsis thaliana*, under different light intensities [where 100% light intensity is 3,120 ± 87 µmol(photons) m^{-2} s^{-1}; *see* Table 1], the curves are averages of 15 replicates ± SE.

lower (Table 2). This problem is, most likely, related to difficulties in getting accurate values of the extrapolated $F_{t\to0}$ at high light intensities (*see* below).

Further, we have compared at each light intensity the OJIP transients measured with *Handy PEA* (Fig. 2) and *FluorPen* (Fig. 3), after normalization to F_o, to determine the effect of differently obtained F_o values on the normalized curves. These two figures show that the curves normalized to $F_o = F_{50\mu s}$ are clearly distorted as the light intensity increases, compared to those normalized to $F_o = F_{20\mu s}$ (and $F_{t\to0}$), since a larger fraction of PSII RCs have the Q_A reduced at 50 μs than at 20 μs, while Q_A, in principle, must be oxidized in all PSII RCs at t = 0. Moreover, the transients measured with blue light (*FluorPen*) are much more affected by normalization to $F_o = F_{50\mu s}$ (and $F_o = F_{20\mu s}$) than those measured with red light (*Handy PEA*), due to higher effective light intensity of the blue light, as shown in Fig. 1, which leads to an increased fraction of PSII RCs with reduced Q_A.

Fig. 4 shows the light intensity dependence of the peak F_P, the initial fluorescence F_o (= $F_{t\to0}$), and the F_v/F_P ratio, obtained from the OJIP curves measured on intact leaves of *Arabidopsis*. The fluorescence intensities, measured with *FluorPen*, are somewhat higher than in *Handy PEA*, which

is expected, since *FluorPen* and *Handy PEA* use different cutoff filters for the fluorescence (for details, *see* 'Materials and methods'). Therefore, in the following discussion, we compare the relative increase of F_P and F_o at five different illumination levels [100%, and ~ 80, 50, 30, and 20% light, where 100% is 3,120 ± 87 and 2,890 ± 70 μmol(photons) m⁻² s⁻¹ for *FluorPen* and *Handy PEA*, respectively; *see* Table 1]. The relative increase of F_P, in the data obtained with both instruments, was (roughly) proportional to the increase in light intensity: *i.e.*, as the illumination increased approximately 1.5, 2.5, 4, and 5 times, the corresponding F_P values increased essentially in the same manner (*see* Figs. 4 and 5). This linear dependence of F_P with light intensity shows that all high light intensities used in this study, for both *Handy PEA* and *FluorPen*, were saturating (*i.e.*, all Q_A molecules were reduced at the P level, and thus, $F_P = F_m$; Kitajima and Butler 1975). Further, the relative increases in the three types of F_o values are shown in Table 2; these data show that F_o, measured as $F_{50\mu s}$, must be in error for higher light intensities since it increases much more than the well-known linearity predicts. However, the data for $F_{20\mu s}$ is quite linear and, thus, more reliable, while, surprisingly, $F_{t\to0}$ decreased at higher light intensities (Table 2). This is, perhaps, because
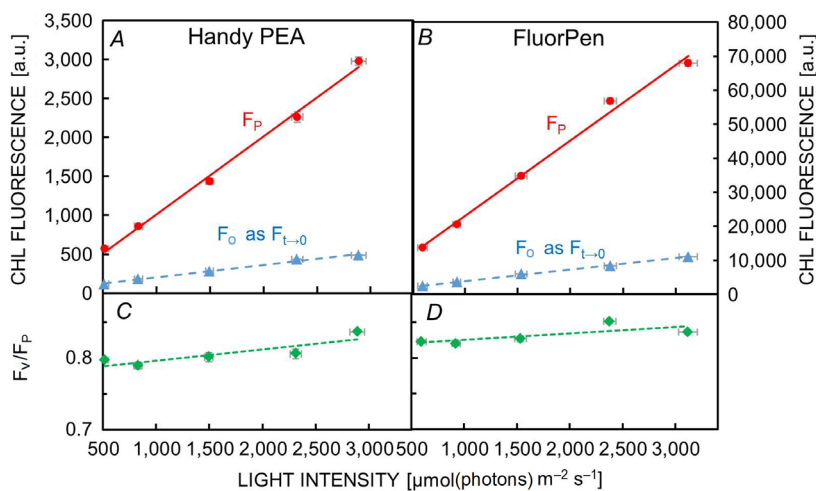

Fig. 4. The 'initial' (Chl *a*) fluorescence (F_o as extrapolated to time zero, $F_{t\to0}$), the peak (F_P), as well as the F_v/F_P ratios, calculated from the OJIP curves, as measured with *Fluor Pen* and *Handy PEA* on intact leaves of wild type *Arabidopsis thaliana* at different excitation light intensities [where 100% is 2,890 ± 70 and 3,120 ± 87 μmol(photons) m⁻² s⁻¹ for HP and FP, respectively; *see* Table 1]. The fluorescence data were fitted with linear trendlines. (*A*) F_o and F_P data from *Handy PEA* (excitation λ = 650 nm). (*B*) F_o and F_P data from FluorPen (excitation λ = 470 nm). (*C,D*) F_v/F_P ratios measured with *Handy PEA* and *FluorPen*, respectively. The data points are averages of 15 replicates ± SE.
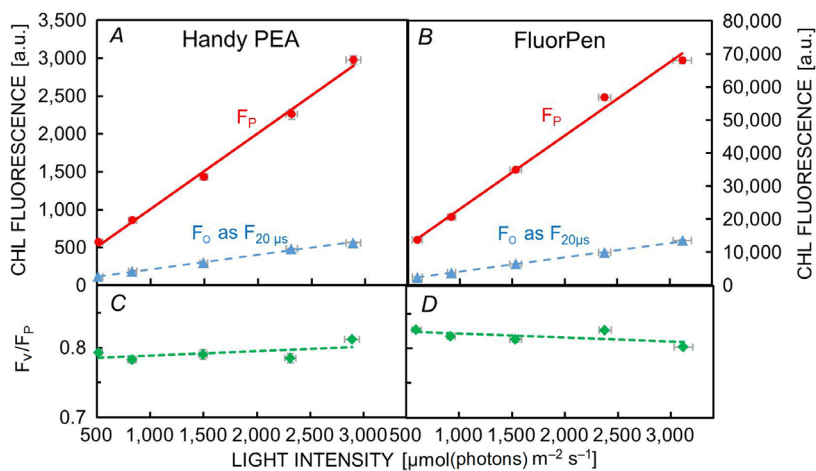
Fig. 5. The initial (Chl *a*) fluorescence ($F_o = F_{20\mu s}$), the peak (F_P), as well as the F_v/F_P ratios calculated from the OJIP curves measured, with *FluorPen* and *Handy PEA*, on intact leaves of wild type *Arabidopsis thaliana* at different excitation light intensities [where 100% light intensity is 2,890 ± 70 and 3,120 ± 87 μmol(photons) m⁻² s⁻¹ for HP and FP, respectively; *see* Table 1]. The fluorescence data were fitted with linear trendlines. (*A*) F_o and F_P data from *Handy PEA* (excitation λ = 650 nm). (*B*) F_o and F_P data from *FluorPen* (excitation λ = 470 nm). (*C,D*) F_v/F_P ratios measured with *Handy PEA* and *FluorPen*, respectively. The data points are averages of 15 replicates ± SE.

Table 2. The relative increases (± SE) of three different F_o values with increasing light intensity: F extrapolated to t = 0; F at t = 20 μs; and F at t = 50 μs. Chlorophyll *a* fluorescence OJIP transients were measured with *FluorPen* (FP) and *Handy PEA* (HP) fluorometers, using 20-min dark-adapted intact bottom leaves of 4-week-old wild type *Arabidopsis thaliana*, at five different light intensities (*see* Table 1), with the approximate relative light increases, noted in the left column, where 1 is for the lowest light intensity used. Data are averages of 15 replicates ± SE.

Relative light increases (based on data in the FP and HP manuals)		Relative increases of F_o with increasing light intensity		
		F extrapolated to t = 0	F at t = 20 μs	F at t = 50 μs
1.5	FP	1.52 ± 0.01	1.55 ± 0.01	1.58 ± 0.01
	HP	1.56 ± 0.02	1.58 ± 0.02	1.64 ± 0.02
2.5	FP	2.47 ± 0.02	2.60 ± 0.02	2.82 ± 0.02
	HP	2.43 ± 0.03	2.52 ± 0.03	2.64 ± 0.04
4.0	FP	3.48 ± 0.03	3.96 ± 0.03	4.58 ± 0.04
	HP	3.75 ± 0.05	4.08 ± 0.05	4.50 ± 0.06
5.0	FP	4.58 ± 0.04	5.40 ± 0.04	6.50 ± 0.05
	HP	4.20 ± 0.05	4.78 ± 0.06	5.37 ± 0.13

in our extrapolation to t = 0, we have not considered possible sigmoidicity of the O–J phase, related to PSII excitonic connectivity (Joliot and Joliot 2003, Joly and Carpentier 2009, Stirbet 2013) but, instead, used a linear fitting. In our study, our focus was (and is) on comparative measurements with the *FluorPen* (blue excitation), *Handy PEA* (red excitation). The overall problem of true (and real) 'F_o' requires further research, especially because it is crucial to obtain correct values of F_v/F_m (or F_v/F_P), and, thus, of the quantum yield of photosynthesis.

Further, the F_v/F_P ratios, obtained by using $F_o = F_{t \to 0}$, increased with the intensity of light for *Handy PEA* (Fig. 4C), with values of 0.797 ± 0.003 (for ~ 20% light) and 0.836 ± 0.001 (for 100% light); for *FluorPen*, they also increased (*see* Fig. 4D) from 0.823 ± 0.0018 (~ 20% light) to 0.837 ± 0.0016 (100% light). On the other hand, the F_v/F_P ratios for *Handy PEA*, calculated with $F_o = F_{20\mu s}$, also increased with the intensity of light (from 0.793 ± 0.003 for ~ 20% light, to 0.812 ± 0.0016 for 100% light; *see* Fig. 5C), while they slightly decreased for *FluorPen* (from 0.827 ± 0.0018 to 0.802 ± 0.0018; *see* Fig. 5D).

If we accept 0.83 as the highest F_v/F_P ratio, when using $F_o = F_{t \to 0}$, for both *FluorPen* and *Handy PEA*, the 'true' F_v/F_P value corrected for the PSI fluorescence (using 30% as contribution of PSI to F_o; *see* Pfündel 1998, Rappaport *et al.* 2007) would be 0.88 (*see* Table 2 in Stirbet and Govindjee 2012), which is close to 0.91 (both ~ 0.9), obtained by Wientjes *et al.* (2013) for plants acclimated to high light. Note that due to different detection windows of the two instruments, used here (*see* 'Materials and methods'), the contribution of PSI fluorescence to F_o is lower in *FluorPen* than in *Handy PEA*, and thus, the uncorrected F_v/F_m ratios determined with *FluorPen* at different light intensities (*see* Figs. 4 and 5) were slightly higher than those determined with *Handy PEA*.

Discussion

All the experimental results, taken together, show that the OJIP transients measured under five different light intensities (*see* Table 1) on intact leaves of *Arabidopsis*

thaliana plants grown under controlled (normal) conditions (Fig. 1), as well as the values of F_o, F_P, F_v/F_P, and F_v/F_o obtained from these data (Fig. 4), are somewhat different when a *Handy PEA* (using red excitation) or a *FluorPen* (using blue excitation, and slightly higher light intensity) was used. Our results on OJIP Chl *a* fluorescence transients, with two different highly-used instruments, presented in this paper (Figs. 1–4), emphasize two major points: (*1*) Choice of F_o is crucial – F_o at 20 μs is much more reliable than that at 50 μs because, in all likelihood, there is higher concentration of reduced Q_A at the longer time; although the use of F_o extrapolated linearly from 80 μs to zero time, as we did here, is, in principle, better, further experiments are needed on this issue. (*2*) The wavelength of excitation is important; because of slightly higher excitation light and absorptance (absorption), by leaves, of 470 nm (blue) light, used in *FluorPen*, than of 650 nm (red) light (*cf.* McClain and Sharkey 2020), the 'artefact' for 'F_o' is higher in the former. This seems obvious from the higher initial slope of the O–J phase measured with *FluorPen* than with *Handy PEA*, for almost all the light intensities used. The F_v/F_P ratios, calculated by using $F_o = F_{t \to 0}$, increased with light intensity for both *FluorPen* and *Handy PEA*, but with a higher slope for *Handy PEA* (Fig. 4C,D); their highest values being the same (0.836–0.837), characteristic of many normal (non-stressed) plants. However, as mentioned earlier, and is well known, Chl fluorescence from PSI influences the F_v/F_P ratio, and, thus, a correction must be made to determine the correct F_v/F_P, to obtain the true quantum yield of PSII (*see e.g.*, Pfündel 1998, 2021; Wientjes *et al.* 2013, Pfündel *et al.* 2018).

We note that different colors of excitation light influence various reactions in photosynthetic samples. For example, the action spectra of PSII photoinhibition, measured on isolated thylakoids (Hakala *et al.* 2005), as well as on *Arabidopsis* leaves (Sarvikas *et al.* 2006), show a relatively similar photoinhibition at 470 nm and 650 nm. However, the relative PSII and PSI contributions to the total fluorescence vary with excitation wavelength, as they have different pigment composition, partly due to the presence

of different chlorophyll–carotenoid protein complexes (*see e.g.*, Lichtenthaler *et al.* 1982a,b; Boichenko *et al.* 1998, Santabarbara *et al.* 2019). Moreover, since PSI has practically no variable fluorescence (Pfündel 1998, Franck *et al.* 2002; *see*, however, Lazár 2013, Schreiber and Klughammer 2021), its contribution to F_o is higher (~ 30–50%) than that to F_P (< 10%) (Pfündel 1998, 2021; Pfündel *et al.* 2013). Thus, the F_v/F_m ratio also varies with the excitation wavelength (Pfündel 2009), being positively correlated with the PSII/PSI absorption ratio, a maximum in the action spectrum of F_v/F_m indicating a higher light absorption by PSII relative to PSI.

On the other hand, measurements of Chl fluorescence profiles and gradients of absorbed light of different colors in spinach leaves have shown that blue light is absorbed in the first 150-μm layer beneath the irradiated surface, followed by red light (200 μm), and then green light (300 μm) (Vogelmann and Han 2000, Evans 2009, Sharkey 2020). This suggests that the blue light, used in *FluorPen* (*FP-110*), may not only have a higher excitation light and leaf absorptance (absorption) than the red light used by *Handy PEA*, but it also samples a different population of chloroplasts in the leaf than the red light. Chloroplasts closer to the surface see more light, and behave more like sun-acclimated chloroplasts, with increased Chl a/b ratio, and greater cytochrome b_6/f and Rubisco content per Chl than the chloroplasts located deeper in the leaf (Terashima and Inoue 1985). The fact is that blue light is absorbed in the light-harvesting Chl protein complexes (LHCPs) in the thylakoid membranes by both the yellow carotenoids and by the two chlorophylls. In contrast, red light is only absorbed by the two chlorophylls and therefore penetrates deeper into the leaf, *i.e.*, also in deeper chloroplast layers not reached by blue excitation light.

Furthermore, due to the overlap with the *in vivo* absorption band of the Chl–protein complexes, the reabsorption of some of the emitted Chl a fluorescence decreases the F_{690} (red) band to a much higher degree than the F_{730} (far-red) band (Gitelson *et al.* 1998, for chloroplasts, *see* Govindjee and Yang 1966). Thus, the emission spectra induced by red light, which penetrates deeper into the leaf, has a fluorescence peak mainly in the far-red, near 735 nm, since a large portion of the F_{690} is reabsorbed on its way from deeper leaf layers to the leaf surface (*see e.g.*, Rinderle and Lichtenthaler 1988). However, Chl fluorescence emission spectra, obtained by excitation with blue light, have a distinctly higher peak at 690 nm, and a lower peak or shoulder at 735 nm. These observations then indicate that blue light, although it may be somewhat less effective in photosynthetic quantum conversion, as compared to red light, gives a higher intensity of Chl fluorescence, because the latter is less reabsorbed since it predominantly comes from the upper chloroplasts closer to the leaf surface. This fact seems to be a major cause for the differences in the blue and red light induced OJIP transients of leaves, as presented here.

Concluding remarks

In conclusion, our measurements of the OJIP transients on the leaves of *Arabidopsis thaliana* plants with *FluorPen*

(using blue excitation light; λ = 470 nm), compared to those with *Handy PEA* (using red excitation light; λ = 650 nm), indicate that blue light has a higher leaf absorptance (absorption) than the red light (Fig. 1), which is supported by, *e.g.*, data of McClain and Sharkey (2020). In principle, the increased intensity of the blue light should have no effect on the F_v/F_m ratio. However, the three different values of 'F_o' (*i.e.*, $F_{t\rightarrow0}$, $F_{20\mu s}$, and $F_{50\mu s}$) affect differently the normalization of the OJIP curves (Figs. 2, 3), and the values of the quantum yield of PSII photochemistry (inferred from the F_v/F_m ratio) (Figs. 4C,D; 5C,D). These 'errors' were, as expected, higher for $F_{50\mu s}$ than $F_{20\mu s}$ and $F_{t\rightarrow0}$, as well as higher for blue light (*FluorPen*) than red light (*Handy PEA*) due to its increased excitation light intensity and of the leaf absorptance (absorption), which induce a higher fraction of PSII RCs with Q_A reduced at higher light intensities. Thus, it is important to be aware of these differences in Chl a fluorescence induction measurements with the two instruments, used here, which use different excitation light. However, it is acceptable to use the same instrument for comparing photosynthetic activities between different samples.

Measurements of Chl a fluorescence transients, used here, provide information on single leaf spots. A more powerful and advanced technique is the imaging of Chl a fluorescence of a larger leaf area or whole leaves providing the individual fluorescence information on several hundred or thousand leaf pixels. We note that Küpper *et al.* (2019) have shown that the direct method of Chl a fluorescence transient measurement, used here, and in the literature, are supported by their data on direct fast imaging with a new version of the macroscopic *FluorCam* instrument (*Photon Systems Instruments*), which uses for excitation the same type of blue LEDs and similar cutoff filters for the fluorescence detection as *FluorPen FP-110*, used here. However, compared to nonimaging measurements, the fluorescence imaging systems enable a much more accurate estimation of the extent and development of stress, since they also allow evaluation of the heterogeneity of measured parameters and the detection of smaller or larger gradients over the leaf area.

Chl a fluorescence imaging systems have been used since the mid 1990s (*see e.g.*, Lang *et al.* 1994, Edner *et al.* 1995, Genty and Meyer 1995, Lichtenthaler and Miehé 1997, Lichtenthaler and Babani 2000, Lichtenthaler *et al.* 2005, Küpper *et al.* 2007, Pérez-Bueno *et al.* 2019). Blue light excitation systems have been used in studies with several imaging systems (*see e.g.*, Lichtenthaler *et al.* 2000, Nedbal *et al.* 2000, Barbagallo *et al.* 2003, Chen *et al.* 2009). Further, an orange/white LED excitation system was used by Cen *et al.* (2017). Multicolor fluorescence imaging systems, including the measurement of the blue and green fluorescence of leaves, as well as the red and far-red Chl a fluorescence, using UV-A radiation (340 to 398 nm) as excitation source were used, *e.g.*, by Lang *et al.* (1994), Lichtenthaler *et al.* (1996), Buschmann and Lichtenthaler (1998), Sowinska *et al.* (1999), and Pérez-Bueno *et al.* (2016). Some of these instruments also allow the user to apply special blue or red filters (with white light as excitation source) to obtain a higher Chl fluorescence yield, using blue or red excitation as compared to UV-A

excitation. On the other hand, Langsdorf *et al.* (2000), Heisel *et al.* (1997), and Sun *et al.* (2020) have shown that Chl *a* fluorescence studies including fluorescence imaging can be used in 'nitrogen management', since they observed a clear relation between Chl *a* fluorescence images and nitrogen status of the plants. In addition to fluorescence intensity measurements, we recommend the use of lifetime of fluorescence imaging as well since it allows one to measure the true quantum yield of PSII (*see* Holub *et al.* 2000, 2007; Wientjes *et al.* 2013).

In conclusion, the differences in the fast Chl fluorescence induction on *Arabidopsis thaliana* plants, obtained with *FluorPen FP-110* (blue excitation ~ 470 nm) with those with *Handy PEA* (red excitation, 650 nm), are mainly due to increased effectiveness of the blue light in Chl fluorescence coming from the outer upper layers of the leaf and to the deeper penetration of the red light also into lower leaf and chloroplast layers, and to about 10% higher light intensity in *FluorPen* (*see* Table 1). However, the F_v/F_m ratios measured with these instruments were comparable, when using $F_{20\mu s}$ and $F_{t\to0}$. We emphasize that some of the issues discussed in this report are also important for a proper interpretation of the Chl *a* fluorescence results obtained in imaging experiments, and *vice versa*.

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