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Bio-optical inferences from chlorophyll *a* fluorescence: What kind of fluorescence is measured in flow cytometry?

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Abstract

A comparison is made of the in vivo Chl *a* fluorescence per cell measured by the flow cytometer (F_{cyt}) and dark-adapted bulk fluorescence measured in a standard field fluorometer for the marine cryptomonad *Chroomonas* sp. (clone Chang 2). The bulk fluorescence protocol estimated the levels of the minimum (F_0) and maximum (F_{max}) fluorescence yields that are exhibited depending on the redox state of the photosystem II reaction center. Both F_0 and F_{max} are known to be functions of cell irradiance history. During the illumination of control samples at growth irradiance (40 μmol quanta m^{-2} s^{-1}), F_0, F_{max}, and F_{cyt} (EPICS V) all increased by about the same proportion. After exposure to photoinhibiting irradiance (1,700 μmol quanta m^{-2} s^{-1}), F_{max} decreased and F_0 increased. Parameters of the photosynthesis-irradiance curve verified that photoinhibition had occurred, indicating less activity at all irradiances. In contrast to bulk fluorescence measurements, relative changes in F_{cyt} in response to strong-irradiance treatment were much smaller than changes in F_0, and F_{max}. We conclude that this is because F_{cyt} is intermediate between F_0 and F_{max}. Multiple regression analyses suggest that, under the flow cytometry conditions used, F_{cyt} exhibits ~20% enhancement above F_0, i.e. an average of 20% of the increase from F_0 to F_{max}. Time scales of photosystem II primary photochemistry are consistent with this amount of fluorescence enhancement occurring over the residence time of the cell in the laser beam. These results suggest caution in using oversimplified interpretations of F_{cyt}. The enhancement effect should also be considered in other instances where fluorescence is excited by a brief saturating flash, for example, some types of in situ fluorometers.

The flow cytometer (FCM) is a tool of great potential for ecological and physiological study of natural populations of phytoplankton (Yentsch et al. 1983). Instruments such as the Coulter EPICS V can quantify and sort cells with specific characteristics from a mixed sample. One of the primary characters used for separation is the type of light-harvesting pigments that sensitize in vivo Chl *a* fluorescence. The main source of this fluorescence is a single pigment-protein complex, photosystem II (PS II). Only a small number of the photons absorbed by PS II are re-emitted as fluorescence (the fluorescence *yield*); most are either used for photosynthesis or converted to heat.

The fluorescence yield of PS II in vivo is not constant—a situation that can be regarded as unfortunate or advantageous depending on one's point of view. When fluorescence is being used as an indicator of pigment concentration, variability in fluorescence yield creates problems (Lorenzen 1966; Heaney 1978; Cullen 1982). On the other hand, fluorescence yield changes in response to, and is an indicator of, the phytoplankter's environment. One important environmental parameter is irradiance, to which responses occur on several time scales (*see* Falkowski and Kiefer 1985). In general, light above a certain intensity results in a decrease in fluorescence yield. At moderate irradiances and short (min) time scales, the decrease is brought about by several mechanisms that decrease the "effective" optical cross-section of PS II (Kiefer 1973; Vincent 1980; Sakshaug et al. 1988). At high irra-

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Significant symbols

α	Slope of the photosynthesis-irradiance curve at zero irradiance, g C (g Chl a)$^{-1}$ h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$
β	Parameter describing the decrease in photosynthesis at photoinhibiting irradiance, g C (g Chl a)$^{-1}$ h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$
F_{cyt}	Fluorescence yield of cells in the flow cytometer, dimensionless
F_0	Minimum fluorescence yield, dimensionless
F_{max}	Maximum fluorescence yield, dimensionless
F_{var}	Variable fluorescence yield ($=F_{max} - F_0$), dimensionless
I_b	Irradiance at which the initial slope of the photoinhibited portion of the photosynthetic curve extrapolates to zero, $I_b = P_s/\beta$, μmol quanta m^{-2} s^{-1}
P_{max}	Maximum rate of photosynthesis, g C (g Chl a)$^{-1}$ h^{-1}
P_s	Theorctical maximum rate of photosynthesis in the absence of photoinhibition, g C (g Chl a)$^{-1}$ h^{-1}
PPFD	Photosynthetically active photon flux density, μmol quanta m^{-2} s^{-1}

diances and/or longer illumination periods (min–h) there are more profound reductions in yield caused by damage to PS II and loss of photochemical capacity (i.e. photoinhibition sensu Vincent et al. 1984; Neale and Richerson 1987; Neale 1987). Nutrient limitation also causes changes in fluorescence yield due to effects on photochemistry (Kiefer 1973; Cleveland and Perry 1987).

The variability of fluorescence on min–h time scales can be used to advantage in applications to biological oceanography and limnology. Processes important to phytoplankton can have rapid time scales; for example, vertical mixing in the surface layer (epilimnion) can be associated with shifts from surface to 1% irradiance in 30 min (cf. Denman and Gargett 1983; *see also* Neale 1987). Fluorescence yield changes have been related to environmental conditions in a number of spatial and temporal series (e.g. Harris 1980; Abbott et al. 1982; Denman and Gargett 1988). Flow cytometry could be useful in examining single-cell fluorescence in similar studies. However the application of in vivo fluorescence as a bio-optical tool must also take into account that fluorescence yield can change even more rapidly than the time scale of minutes and can, in fact, vary at the "observational" time scale of seconds or faster. Thus, the information content of a fluorescence measurement may depend strongly on instrument configuration.

We report here on how short time-scale variations in fluorescence yield influence the comparison of FCM measurements with those of other fluorometers. The fast variations discussed here occur in the PS II complex during primary photochemistry and can be delimited by two levels of fluorescence yield. The minimum fluorescence yield (F_0) is exhibited by an algal cell when a low excitation intensity is used. Under these conditions photochemistry keeps pace with light absorption and PS II centers remain open. However, when the rate of photon arrival to the reaction center greatly exceeds the rate at which photons can be used for photochemistry, more energy is dissipated by fluorescence and fluorescence yield rises to a maximum, F_{max}. These two fluorescence components can be used to probe the photoadaptive state of the cell, with F_0 indicative of the functional antenna size and F_{max} responsive to both antenna size and photochemistry. (Terms defined in list of symbols.)

Physiological responses to strong irradiance have different effects on F_0 vs. F_{max}. The use of FCM to infer the light history of individual cells in natural water samples depends on knowing what "kind" of fluorescence is being measured. For example, under bright light an alga will decrease functional antenna size—an effect which is rapidly (0–10 min) reversed by dark adaptation (e.g. Vincent et al. 1984). The same bright light may damage the PS II reaction center (photoinhibition) and reduce photochemistry—an effect which is not reversed by short dark adaptation. Thus, exposure to bright light followed by dark adaptation will have a dramatically different effect on F_0 vs. F_{max}. The low excitation intensity beam of the typical benchtop fluorometer excites a yield that is close to F_0; such fluorescence has been denoted "F" or "F_a" (Harris 1980; Vincent 1980; Cullen 1982). A yield approximating F_{max} can be obtained in the same instrument by blocking photochem-

istry with the herbicide DCMU—a measurement termed "F_D" or "F_b." The F_{max} yield is also obtained after illumination with a very intense flash or short duration laser beam (Falkowski et al. 1986).

When an algal cell is placed under intense illumination, there is a "fluorescence induction" as yield increases from F_0 to F_{max}. The fluorescence induction curve has been used as a measure of phytoplankton photosynthetic characteristics (Bates and Platt 1984; Neale and Melis 1986); however, little effort has been made to resolve how these kinetics influence other, nonspecialized, measurements of fluorescence (Harris 1980). The rate at which the yield rises depends on PS II photochemistry which, after illumination with an intense laser beam, has picosecond to microsecond time scales (reviewed by Glazer and Melis 1987). Such illumination conditions exist in the flow cytometer, but it is unknown what yield is applicable to measurements of algal cells, particularly for those algae containing phycoerythrin. A previous report on isolated higher plant chloroplasts has suggested that FCM measures a pure F_0 yield (Ashcroft et al. 1986). The results reported here on a marine cryptomonad suggest that FCM fluorescence is intermediate between F_0 and F_{max}.

Methods

Experimental organism—A culture of the marine cryptomonad *Chroomonas* sp. (clone Chang 2) was grown in F/2 media at 20°C under fluorescent lighting (photosynthetically active photon flux density, PPFD = 40 μmol m^{-2} s^{-1}; 14 : 10 L/D). The lights came on at 0700 hours local time, and treatments started after 0900 hours.

Instrument configurations—Flow cytometric analysis was accomplished with an EPICS V flow cytometer (Coulter Electronics) equipped with a 5-W argon laser. Laser power was 250 mW at the 488-nm excitation wavelength. Emissions from the autofluorescing pigments were split by a 590-nm dichroic filter and measured within the optical range of 560–590 nm for phycoerythrin (PE) emission and at >665 nm for Chl emission. Bulk Chl *a* fluorescence was measured with a Turner Designs 10-005R fluorometer fitted with a Corning 5-60 excitation filter and 2-64 emission filter.

"Phycoerythrin" fluorescence was measured using a lamp with a 546-nm emission line for excitation and a combination of 4-97 and 3-66 emission filters. Unless otherwise indicated all samples were dark adapted for 5 min before measurement. After an initial reading (F_0) was taken, a saturated solution of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in ethanol was added to a final concentration of 10 μM. The sample was returned to the fluorometer and a second reading was made once fluorescence rose to a maximum level (F_{max}). The difference between these two fluorescences levels is the variable fluorescence, $F_{var} = F_{max} - F_0$.

Instrument intercalibration—We used a novel method to intercalibrate the flow cytometer and the benchtop fluorometer which verified that instrument responses were proportional. The instruments were tested in the excitation and emission configurations used to measure both Chl and PE fluorescence. Suspensions of 10-μm fluorospheres (Coulter Electronics, Inc.) with designated intensities of 25, 50, and 100% "bright" were measured in the Turner Designs instrument, then the particle densities (variable between suspension) were determined by a Coulter counter. Fluorospheres have a wide emission spectrum with includes both the PE and Chl emission range. A relative fluorescence per bead was computed and compared to the mean fluorescence per particle measured with the same standards on the EPICS.

Photosynthesis and curve fits—Light-dependent rates of $^{14}CO_2$ assimilation per unit Chl *a* were determined with a photosynthetron (Lewis and Smith 1983), and parameters of the photosynthesis-irradiance (*P-I*) curve were estimated by nonlinear regression fitting of the equation

$$P(I) = P_s[1 - \exp(-\alpha I/P_s)] \times \exp(-\beta I/P_s)$$

(Platt et al. 1980). Asymptotic estimates of parameter confidence intervals were calculated with the SAS statistical package. An estimate was also made of the maximum realized photosynthetic rate (P_{max}) (Platt et al. 1980), with confidence intervals esti-

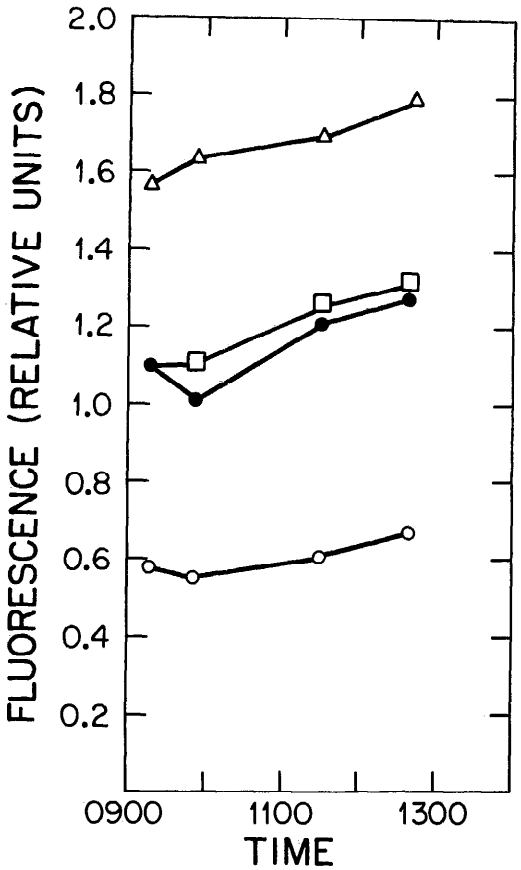

Fig. 1. Variation in fluorescence parameters of *Chroomonas* sp. (Chang 2) during the light period. The in vivo Chl fluorescence measured by the Turner Designs model 10 fluorometer is plotted as output in volts: F_0, i.e. steady state fluorescence of dark-adapted cells—O; F_{max}, i.e. fluorescence in the presence of 10 μM DCMU—△. Normalized fluorescence per cell measured by the EPICS V flow cytometer is plotted in relative units ("mean channel number" with channel 100 = 1.00): Chl fluorescence per cell—□; phycoerythrin fluorescence per cell—●. Local time is given; growth lights came "on" at 0700 hours.

mated essentially as described by Zimmerman et al. (1987). Chl *a* content was determined fluorometrically after extraction in ice-cold 90% acetone for 24 h.

High-light incubation—The focused output from a halogen light source was used for high-light treatments. The incident PPFD on the culture sample was 1,700 μmol quanta m^{-2} s^{-1} with a variation of 10% over the surface of the incubation vessel. A 4-cm-thick water filter was used to protect the sample from heating. The culture was gently agitated during exposure and temperature was kept within 1°C of the growth temperature (20°C).

Results

Instrument response to fluorospheres was linearly correlated in both Chl and PE filter configurations. For the Chl fluorescence the equation was EPICS fluorescence per particle (relative units) = −21.4 + 314.8 (mV Turner Designs fluorescence per particle × 10^5), $R^2 = 0.997$, $n = 3$. For the PE fluorescence configuration the intercept was −8.0 and slope 302.1, again with $R^2 = 0.997$. The small intercepts in the linear regression equations indicate that fluorescence measurements of a fixed emission yield should be approximately proportional in both instruments.

Normal growth light regime—Fluorescence and photosynthetic characteristics were monitored in *Chroomonas* sp. during maintenance of control cultures in the normal growth light regime. All measures of Chl and PE fluorescence increased by about the same relative proportion during the light period (Fig. 1). The increase ranged from 15 to 20% over a 3-h period. A much larger increase was observed in measures of the light-saturated rate of photosynthesis. The maximum rate, P_{max}, went from 1.71 to 2.6 g C (g Chl *a*)$^{-1}$ h^{-1}, an increase of 52%. However, the fluorescence ratio F_{var}/F_{max} did not change during this period, and similarly the initial slope of the *P-I* curve, α, was constant at 0.03 g C (g Chl *a*)$^{-1}$ h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$. Photosynthesis by control cells exhibited photoinhibition at irradiances $\gtrsim$500 μmol quanta m^{-2} s^{-1}, with the I_b parameter ($=P_s/\beta$, Platt et al. 1980) ranging from 800 to 900 μmol quanta m^{-2} s^{-1}.

Photoinhibition and recovery—Comparison was then made of the variation in fluorescence parameters during visible light photoinhibition and subsequent recovery in low light. Exposure of *Chroomonas* sp. to ~1,700 μmol quanta m^{-2} s^{-1} PAR resulted in a large drop in F_{max}, whereas F_0 increased initially and then declined (Fig. 2). After 60 min of photoinhibition F_{max} dropped by about 46%, while there was a slight net increase in F_0. Concomitant photoinhibition

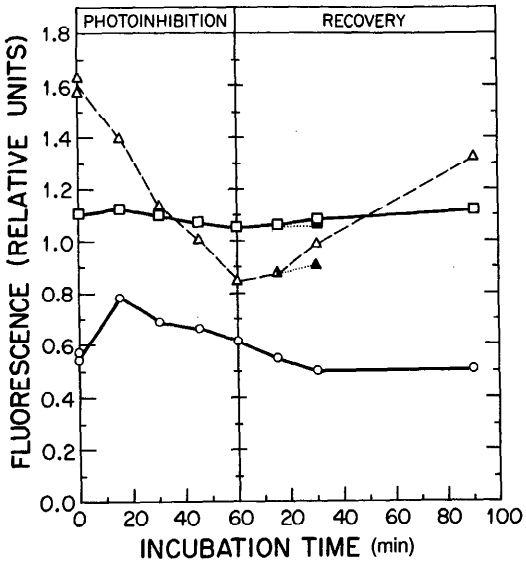

Fig. 2. Variation in Chl fluorescence parameters of *Chroomonas* sp. (Chang 2) during exposure to high irradiance (photoinhibition) or after return to low light (recovery). High irradiance (~1,700 μmol quanta m^{-2} s^{-1}) was maintained for 60 min before the light was extinguished and incubation was continued in ambient light (~10 μmol quanta m^{-2} s^{-1}). Symbols as for Fig. 1. Filled symbols indicate samples from parallel dark incubations; all other samples were dark adapted for 5 min before measurement. Note that when F_0 and F_{max} vary in opposite directions, there is minimal variation in F_{cyt}.

of photosynthesis was confirmed in the *P-I* measurements (*see below*). When the cell suspension was returned to low light (10 μmol quanta m^{-2} s^{-1}), F_{max} increased and F_0 decreased. After 90 min, F_{max} recovered to 81% of the initial value, although this is only 74% of the F_{max} level in samples from the control cultures taken at the same time (Fig. 1). The level of F_0 also remained below the corresponding culture room controls.

The dramatic, and opposing, shifts in bulk Chl fluorescence parameters contrast with the relatively small variation in Chl fluorescence per cell measured by the flow cytometer (Fig. 2). Mean Chl fluorescence per cell (F_{cyt}) ranged from a minimum of 105 (relative units) to a maximum value of 112; the difference is 6% of the initial mean of 110. These variations are significant; with 1,000 cells counted, the 95% C.I. around the mean is ±2 (relative units). The variation in F_{cyt} does not exclusively reflect F_0

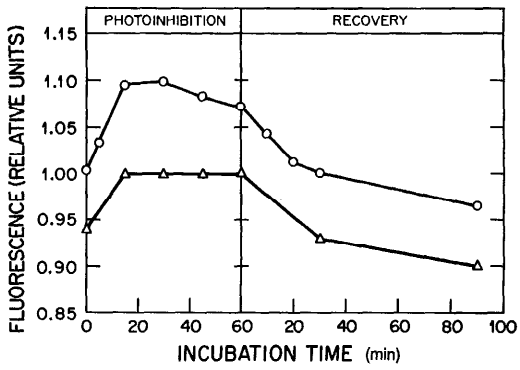

Fig. 3. Variation in PE fluorescence parameters of *Chroomonas* sp. (Chang 2) during exposure to strong irradiance (photoinhibition) or after return to ambient low light (recovery). PE fluorescence measured on the Turner Designs model 10—△; PE fluorescence per cell measured on the EPICS V flow cytometer—○. (In relative units as described for Fig. 1.)

or F_{max}. The initial increase in cytometry fluorescence seems to parallel the increase in F_0 during the first few minutes of high light exposure. However, during recovery F_{cyt} increased, similar to F_{max} and contrary to the decrease in F_0.

Strong-irradiance exposure resulted in an increase in PE fluorescence which was reversed during subsequent low-light recovery (Fig. 3). In contrast to Chl fluorescence, the changes in PE fluorescence measured on the FCM closely tracked bulk PE fluorescence measured with the benchtop fluorometer. Each fluorescence variable increased by about 24% in normal growth controls by the end of the treatment period. Overall, there was a strong proportional relationship between FCM PE fluorescence per cell and bulk PE. The linear regression had an r^2 of 0.96 (n = 8) and the intercept was not significantly different from zero. Increases in PE fluorescence due to stress have also been reported for phycoerythrin-containing marine *Synechococcus* spp. The ratio of FCM measured PE : Chl fluorescence increased when *Synechococcus* WH7803 cultures were deprived of nitrogen (Glibert et al. 1986). The PE : Chl ratio was also negatively correlated with P_{max} for photoinhibition and recovery of *Chroomonas* ($r = -0.83$, $P < 0.05$, $n = 6$); a similar relationship has been described for *Synechococcus* WH7803 and WH8018 (Barlow and Alberte 1985).

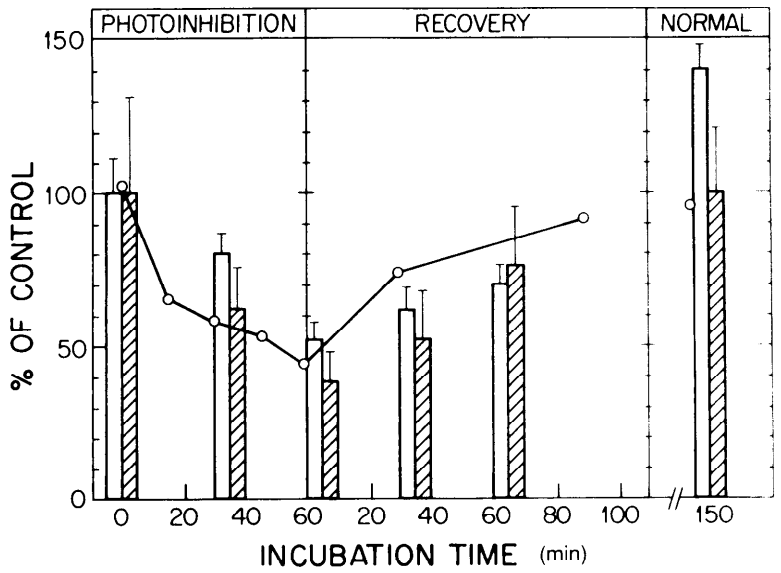

Fig. 4. Relationship of fluorescence parameters to the parameters of the photosynthesis-irradiance curve of *Chroomonas* sp. (Chang 2) during high-light exposure (photoinhibition), after return to ambient light (recovery), and in a parallel control culture that was maintained in growth conditions during the above treatments (normal). Open bars—maximum photosynthetic rates (P_{max}); hatched bars—low-light response (α); extending bar—the width of the parameter 95% C.I. based on asymptotic standard errors; (—○—)—the ratio of F_{var}/F_{max}. Fluorescence and α plotted as percent of time = 0 sample, P_{max} as percent of a control value estimated from interpolation between P_{max} at time = 0 and P_{max} in the parallel control as indicated in the normal culture.

Exposure of *Chroomonas* sp. to high light resulted in a pronounced loss in photosynthetic performance over a wide range of irradiances, although the relative decrease was greater for the low-light response (α) vs. the light-saturated photosynthetic rate (P_{max}). The decrease in dark-adapted variable fluorescence (F_{var}) relative to F_{max} was a good indicator of photoinhibition effects on the photosynthetic parameters (Fig. 4). The ratio F_{var}/F_{max} was more strongly correlated with variation in α than with variation in P_{max}. These results are consistent with those of Bjorkman and Demmig (1987) and Demmig et al. (1987); they reported that the F_{var}/F_{max} ratio is closely correlated with changes in the quantum yield of oxygen evolution under CO_2-saturated conditions in higher plants. Also, correlations between a related fluorescence ratio (F_0/F_{max} in our notation) and rates of light-limited photosynthesis have been reported for marine phytoplankton (Prézelin and Ley 1980).

Although F_0 and F_{max} vary in response to photodamage and recovery of photosynthetic activity, F_{cyt} seems unaffected. We suggest that this variable response is not due to an inherent insensitivity of FCM measurement; instead it arises because F_{cyt} is a composite of both F_0 and F_{max} yields (*see below*). This possibility was empirically examined by using multiple linear regression to determine the best predictor equation of F_{cyt} based on F_0 and F_{max} (Table 1). Equations without an intercept were fitted on the assumption of instrument proportionality (*see above*). Equations were fitted with either each variable separately or a two-variable equation with F_0 and F_{var}. We chose F_{var} as the second predictor in order to minimize correlation between independent variables. A significantly better fit (i.e. lower residual mean square) for F_{cyt} was obtained with both F_0 and F_{var} as predictors compared to either fluorescence variable alone. However, no equation without an intercept predicted F_{cyt} with more accuracy than a constant mean value. The best single predictor in an equation with an intercept was F_{var} which had an R^2 of 0.77. The fitted coefficient for F_{var} is about 20% of either the coefficient of F_0 or the constant term

Table 1. Multiple linear regression analysis of Chl fluorescence per cell of *Chroomonas* sp. (Chang 2) measured by the EPICS FCM (F_{cyt}) as dependent on steady state fluorescence of dark-adapted cells (F_0) or steady state fluorescence in the presence of 10 μM DCMU (F_{max}) measured on the Turner Designs model 10 bulk fluorometer. For convenience, in the two-variable regressions the second predictor is $F_{var} = F_{max} - F_0$. The data used include nine observations during the time-course of photoinhibition followed by recovery of *Chroomonas* sp. and three additional observations on cell suspensions kept in a normal growth light regime (cf. Fig. 1). Since equations with and without an intercept term are compared, the relative fit is measured by mean-square residual as opposed to R^2.

Equation	Mean-square residual
(1) $F_{cyt} = 113$ (mean)	72.7
(2) $F_{cyt} = 185 \times F_0$	313.2
(3) $F_{cyt} = 84 \times F_{max}$	537.9
(4) $F_{cyt} = 149 \times F_0 + 33 \times F_{var}$	193.7
(5) $F_{cyt} = 99 + 22 \times F_{var}$	21.1

Discussion

The experimental treatments used here induced major shifts in the fluorescence yield of a cryptomonad alga that were independent of pigment quantity or composition. Instead, the shifts in fluorescence yield depended on the irradiance history. Strong-irradiance exposure photoinhibited PS II in this alga, which resulted in characteristic shifts in F_{max} and F_0. Parallel determinations of *P-I* parameters showed that these changes in fluorescence were correlated with significant variation in photosynthetic performance. However, the variation in Chl fluorescence emission measured by the flow cytometer was relatively small and could not be uniquely identified with either F_0 or F_{max}.

The thesis of this discussion is that F_{cyt} may not be directly comparable to fluorescence measurements from other instruments because the time scale of measurement in the flow cytometer coincides with an important time scale of the variation in fluorescence yield. Fluorospheres have a constant fluorescence yield and gave absolutely proportional responses between instruments, despite $\sim 10^8$ difference in sample residence time and excitation beam intensity (Fig. 5). Similarly, instrumental response was also proportional for PE fluo-

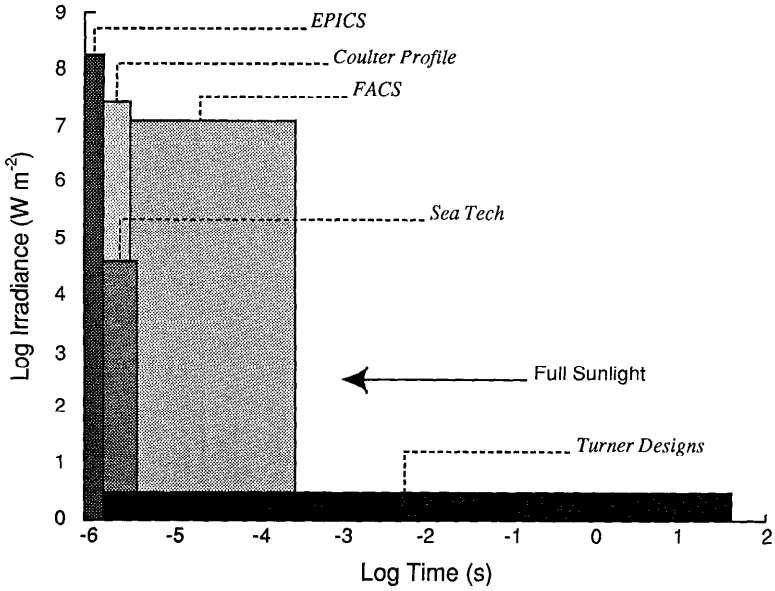

Fig. 5. Excitation irradiance and time of exposure for different instruments that measure fluorescence. Rough estimates calculated from manufacturer's specifications. Note the log scale. For the EPICS flow cytometer, Coulter Profile flow cytometer, and FACS analyzer, fluorescence is measured on the same time scale as excitation exposure, determined by the rate of flow past the sensor. For the SeaTech in situ fluorometer, fluorescence is measured over a longer period than the pulsed excitation. (From Cullen et al. 1988.)

Table 2. Comparison of time scales of flow cytometry measurement and variation of in vivo Chl fluorescence yield in saturating illumination.

Event	Time scale	Derivation	Reference*
Mean interval of photon arrival at PS II	2.5 ns	Typical PS II cross-section (100 A^2) × laser photon flux (625 M quanta m^{-2} s^{-1})	1, 2
Transition to high fluorescent closed reaction center	10–500 ns	Rate-limiting step of PS II photochemistry, i.e. electron donation to P_{680}	3–6
Cell residence time in laser beam	1–10 μs	Illuminated volume/flow rate	1
Time to complete rise to F_{max}	10–25 μs	Kinetic measurements on chlorophyte algae and isolated chloroplasts	3, 5
Return to low fluorescent open reaction center	100–200 μs	Oxidation of PS II electron acceptor, Q_A, by plastoquinone	7

* 1—Coulter EPICS instrument specifications; 2—Ley and Mauzerall 1982; 3—Mauzerall 1972; 4—Butler 1972; 5—Jursinic and Govindjee 1977; 6—Sonneveld et al. 1979; 7—Glazer and Melis 1987.

rescence emission by *Chroomonas*—consistent with our observation that DCMU has negligible effect on PE fluorescence measured on the Turner Designs instrument. In contrast, the in vivo Chl fluorescence yield varies between F_0 and F_{max}. Biophysical studies have established the time scales of PS II primary photochemistry in saturating illumination. The rate-limiting step for the transition from an "open" reaction center (F_0) to a "closed" reaction center (F_{max}) has a time constant of 10–500 ns (Table 2). Fluorescence does not complete the rise to the F_{max} level until several microseconds after saturating illumination has begun (Table 2). Several reasons for the delay have been proposed; one important factor is that high-energy laser flashes lead to the formation of long-lived (μs) quenchers such as carotenoid triplets (Sonneveld et al. 1979).

The fluorescence yield increase for intact cells of *Chlorella* after a single saturating flash (10 ns) shows a rise of 18–42% of F_{var} in the first 100 ns; the remaining rise up to F_{max} occurs over the next 10 μs (Mauzerall 1972). If *Chroomonas* displayed a similar rise during its estimated 1-μs transit time through the laser beam, an average "enhancement" of 25% of the increase between F_0 and F_{max} would occur. The average fluorescence yield would thus be higher than F_0 but lower than F_{max}. Our experimental data on *Chroomonas* have also indicated that F_{cyt} is intermediate between F_0 and F_{max}, and that a 20% average enhancement of F_{var} occurred. The agreement between our empirical estimate and the fluorescence kinetics of *Chlorella* suggests that our characterization F_{cyt} is correct and consistent with the basic dynamics of PS II photochemistry. Many issues remain to be resolved, however, such as how PS II kinetics of cryptomonads and other phycoerythrin-containing algae differ from chlorophytes and the importance of carotenoid triplet formation during continuous illumination by the FCM laser. More studies of fluorescence enhancement kinetics under the conditions of excitation in flow cytometry are necessary.

The conclusion that F_{cyt} lies between F_0 and F_{max} contrasts with the interpretation of FCM fluorescence of isolated chloroplasts and chloroplast membranes by Ashcroft et al. (1986). Sample residence times were on the order of 5 μs in the instrument they used, yet the signal was characterized as reflecting only the F_0 yield component. This interpretation was based on the observation of a maximum yield 25 μs after the saturated pulse, but it fails to take into account that a significant portion the fluorescence yield increase may take place in the first 100 ns (Mauzerall 1972). Carotenoid quenching was not considered either. Also, the time scale of PS II photochemistry could have been considerably longer due to damage to PS II during preparation of subcellular fractions (Conjeaud and Mathis 1980). By comparison, the present study used intact cells in which photoinhibition (which only affects the PS II primary charge separation, Neale 1987) induced opposing changes in F_0 and F_{max}. Such results are more directly applicable to the interpretation of FCM fluorescence as measured in oceanographic and limnological studies.

Finally, what recommendations can be made for the practical application of FCM as a method in the study of ocean bio-optics? The high excitation intensities used by flow cytometers will make it difficult to measure a "pure" F_0 fluorescence yield. The same limitation applies to other types of fluorometers which rely on brief but high-intensity excitation (e.g. the Sea Tech in situ fluorometer). Thus fluorescence results will not only be a function of the cellular pigment content and pigment distribution within the cell, but also of the photochemical activity of the PS II reaction center. It becomes especially important in the comparison of FCM fluorescence between algal cultures or populations in conditions that may affect PS II function such as nutrient limitation (Cleveland and Perry 1987) or high-visible light exposure or exposure to ultraviolet (Neale 1987).

Because F_{max} is responsive to bright light, it might be useful to optimize FCM to measure F_{max}. Careful attention to the kinetics of fluorescence enhancement is required. Extending the residence time of the cell in the beam or positioning a second probe beam downstream to make the fluorescence measurement about 20 μs after the primary laser flash are possible modifications that can be tried (Ashcroft et al. 1986). Varying the residence time between 1 and 10 μs—a range easily obtainable on the EPICS—may substantially affect the nature of the signal being measured, which should be kept in mind when changing the flow rate of the instrument. Other types of instrument modifications may be more difficult. Variation in the "kind" of fluorescence measured also may account for the differences between instruments observed in measuring bright-light responses by *Thalassiosira pseudonana* (Cullen et al. 1988)—the much larger response of the in situ fluorometer being due to lack of dark adaptation.

We have shown that measurements of fluorescence by FCM are dominated by factors which affect the minimum in vivo Chl fluorescence yield (F_0) of the algal cell, but are also influenced by a variable fluorescence yield enhancement which reflects the activity of the PS II reaction center and, under the intense excitation of the FCM laser, increases fluorescence emission to a maximum level (F_{max}). Fluorescence data on the marine cryptomonad *Chroomonas* sp. (Chang 2) suggest that an average enhancement of 20% occurred in measurements with the EPICS V flow cytometer. The presence of this enhancement in the data should be taken into account in interpreting FCM data on algal fluorescence as well as in comparing FCM fluorescence with measurements made with other fluorometric methods.

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