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Photosynthetic Characteristics of Marine Phytoplankton From Pump-During-Probe Fluorometry of Individual Cells at Sea

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Background: Active fluorescence techniques are becoming commonly used to monitor the state of the photosynthetic apparatus in natural populations of phytoplankton, but at present these are bulk water measurements that average all the fluorescent material in each sample. Here we describe two instruments that combine individual-cell ''pump-during-probe'' (PDP) measurements of chlorophyll (Chl) fluorescence induction, on the time scale of 30 to 100 μs, with flow cytometric or visual characterization of each cell.

Methods: In the PDP flow cytometer, we measure Chl fluorescence yield as a function of time during a 150 μs excitation flash provided by an argon ion laser; each particle is subsequently classified as in a conventional flow cytometer. In the PDP microfluorometer, individual cells in a sample chamber are visually identified, and fluorescence excitation is provided by a blue light-emitting diode that can be configured to provide a saturating flash and also a subsequent series of short flashlets. This sequence allows both saturation and relaxation kinetics to be monitored.

Results: Phytoplankton from natural samples and on-deck iron-enrichment incubation experiments in the Southern Ocean were examined with each PDP instrument, providing estimates of the potential quantum yield of photochemistry and the functional absorption cross section for photosystem 2, for either individuals (for cells larger than a few micrometers) or populations (for smaller cells).

Conclusions: Results from initial field applications indicate that single-cell PDP measurements can be a powerful tool for investigating the nutritional state of phytoplankton cells and the regulation of phytoplankton growth in the sea. Cytometry 37:1–13, 1999. © 1999 Wiley-Liss, Inc.

Key terms: active fluorescence; pump-during-probe; single cell; flow cytometer; microfluorometer; photosynthesis; phytoplankton; photosystem 2

Active fluorescence techniques such as pump-and-probe and fast repetition rate (FRR) fluorometry provide information about the functional state of photosynthetic cells. These techniques are based upon time-resolved measurements of chlorophyll (Chl) fluorescence, which comes mainly from photosystem 2 (PS2) and which changes in response to light absorption. In an initial dark-adapted state when reaction centers are ''open'' or capable of accepting excitation energy, fluorescence intensity is low (F_o), while in the light-saturated state, reaction centers are ''closed'' and fluorescence yield is maximal (F_m). The difference between F_m and F_o is the variable fluorescence (F_v), which when normalized to F_m represents the potential quantum yield of photochemistry ($\phi = F_v/F_m$, relative variable fluorescence). F_v/F_m ranges from 0 (indicating nonfunctional reaction centers or dead cells) to about 0.65 for fully functional phytoplankton (2). Other properties which can be investigated with active fluorescence include the functional absorption cross section (σ, which for a given excitation intensity is related to the rate of increase in fluorescence yield), and the turnover time of electron transfer out of PS2 reaction centers (τ, as indicated by the rate of decay back to the dark-adapted fluorescence yield after saturation) (1,2). The photosynthetic parameters can themselves be diagnostic of nutri-

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tional state of the phytoplankton; for example, nitrogen- and iron-limited cells exhibit loss of functional reaction centers, evident through decreasing relative variable fluorescence and increasing functional absorption cross sections (3–5) .

Active fluorescence measurements are typically carried out on bulk samples, with results representing the weighted average of all the different kinds of fluorescent material present, including any detrital particles and dissolved materials that emit red fluorescence in addition to phytoplankton cells. If all the fluorescent materials do not have the same properties, bulk measurements will thus depend on the composition of the community as well as the physiological state of the phytoplankton cells. To investigate heterogeneity in the phytoplankton and other particles and to directly evaluate the contributions of different kinds of phytoplankton to such bulk signals, we developed two techniques to measure active fluorescence on individual phytoplankton cells, using a ''pump-during-probe'' (PDP) approach (6). Our approach is a variation on the FRR technique, and differs mainly in that we use a single pulse of light rather than a series of short flashlets to saturate PS2. As in the FRR technique, the PDP measurements are made using excitation light intensities that cause saturation of fluorescence yield on the time scale of a single turnover of PS2 reaction centers.

Laboratory prototypes of the PDP microfluorometer and flow cytometer demonstrated that active fluorescence measurements could be performed on single phytoplankton cells (6), but these prototypes were of limited usefulness for analyzing natural samples. For example, the microscope-based PDP instrument was very slow to use because of the 2-min dark adaptation period required before each measurement. Similarly, even though the flow cytometer-based instrument measured individual cells, it was useful only for pure cultures since there was no way to identify the subject of each PDP measurement. The present work describes new PDP instruments that have been used at sea to measure photosynthetic characteristics of individual cells and natural populations of phytoplankton.

The major innovations in the PDP microfluorometer are the use of infrared illumination during the location and positioning of a subject cell (which eliminates the need for dark adaptation), the use of a water-jacketed flat capillary microscope slide which facilitates sample changing and temperature control, and the use of a programmable digital light emitting diode (LED) controller, which allows complex excitation flash trains for analysis of fluorescence decay as well as induction. The PDP flow cytometer is a completely new instrument in which each particle passes sequentially through three independently controlled laser beams: an infrared beam for particle detection, a weak 488 nm beam for PDP Chl fluorescence excitation, and a strong 488 nm beam for particle classification by conventional flow cytometric measurements of forward and side light scattering and red and orange fluorescence.

MATERIALS AND METHODS

PDP Microfluorometer

The PDP microfluorometer is based on a modified Zeiss trinocular epifluorescence microscope (Fig. 1). Phytoplankton cells are assayed in a flat glass capillary (Diasys Corp., Waterbury, CT) mounted on a microscope slide so that water can be circulated under the sample chamber for temperature control. The capillary is filled with seawater sample through a silicone tube using a syringe, and both ends of the filled capillary are closed; this prevents sloshing of the sample with ship movement and allows measurements to be made under even severe weather conditions (up to at least 15° rolls). A cell is located and positioned in the center of the field of view using bright-field optics. To maintain the cell in a dark-adapted state, positioning is carried out under infrared (IR) illumination (RG780 filter) detected by a charge coupled device (CCD) camera (Hitachi KP-160 with its IR blocking filter removed) mounted on one of the binocular tubes. An image of each cell measured can be stored digitally. The IR illumination is blocked, and the prism in the trinocular head is shifted out of the light path before the fluorescence induction measurement.

Fluorescence is excited by a pulse from a blue LED (emission 470 nm, NLPB500, Nichia America Corp.) positioned in place of the normal Hg arc lamp. The duration (typically 100–200 μs) and intensity of the blue flash are controlled by a custom circuit based on a 555 timer chip and a field effect transistor, or in later work by a programmable digital function generator (Stanford Research DS345) driving a field effect transistor. The time constant for rise of the blue flash to maximum output is ≈1 μs. Typically the pulse current through the LED (which is rated at 100 mA for a 10 ms pulse) is 300 mA for 150 μs. Use of the digital function generator also allows the decay in fluorescence yield after the attainment of maximum yield to be measured, through a series of short (2–3 μs) ''probe'' flashes at intervals of 100 μs (see Discussion). To monitor the excitation flash, a photomultiplier (PMT) (Hamamatsu 931B) screened by a neutral density filter (ND 2) is placed at the window normally used to view the arc lamp for alignment and focusing. Zeiss filter set 487709 allows blue excitation light to illuminate the sample and red Chl fluorescence to pass up the tube to a second PMT (Hamamatsu R1477). A red bandpass interference filter (680DF40, Omega Optical, Inc., Brattleboro, VT) is placed in front of the fluorescence PMT, and an aperture in a sliding holder below the prism limits the field of view of the PMT to minimize the amount of background signal and to isolate the cell of interest from neighboring cells during the measurement.

Signals from both fluorescence and reference PMTs are amplified (5×) with a 300 MHz preamplifier (SR445, Stanford Research, Inc.), smoothed with a resistor-capacitor chain (time constant = 0.25 μs), and recorded at 20 MHz with a digital oscilloscope (LeCroy 9314L), or in later work at 30 MHz with a 12-bit analog-to-digital

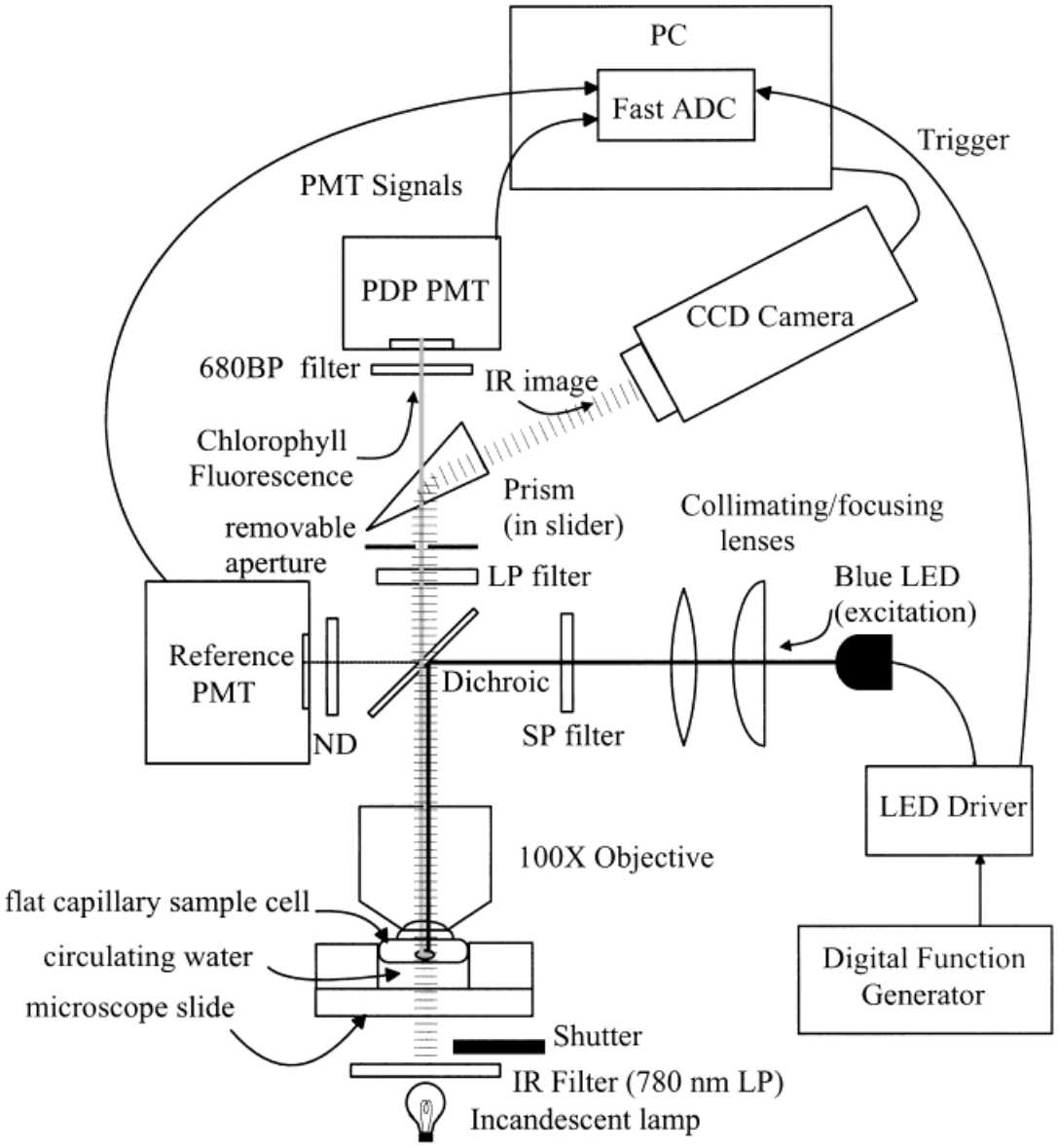


FIG. 1. Schema of the PDP microscope. In early work, the LED was controlled by an analog circuit based on a 555 timer chip, and signals were captured with a digital oscilloscope and subsequently transferred to a personal computer (PC). Longpass, shortpass, bandpass, and neutral density filters are indicated as LP, SP, BP, and ND, respectively.

converter (ADC) board (Gage Scientific 6012) mounted in a personal computer. The oscilloscope or ADC board is triggered by the pulse sent to turn on the LED. The dark current from the PMT (obtained by recording the fluorescence signal before the LED pulse), is subtracted from the signals, as are background signals (obtained by measuring an empty field of view).

A series of 50 time-resolved measurements at 2 Hz is collected for each cell measurement. These data are binned to 1 μs intervals and averaged to increase the signal-to-noise ratio. The induction curve of Chl fluorescence yield is obtained by dividing each fluorescence value by the relative excitation intensity measured at the same time (reference signals). These normalized data are then fitted to a theoretical model (6) to obtain estimates of relative variable fluorescence (ϕ) and the product σI, the rate of trapping of absorbed photons by reaction centers (where I is the absolute excitation light intensity). For preliminary assessments of fluorescence yield decay, the time series of normalized integrated fluorescence from each flashlet was fitted to a two-component exponential model with turnover times τ_1 and τ_2.

PDP Flow Cytometer

The optical part of the flow cytometer is constructed on an optical breadboard (36 × 48 × 2 in thick), and is based on the flow cell and collection lens assembly from a Becton Dickinson (Mansfield, MA) FACScan flow cytom-

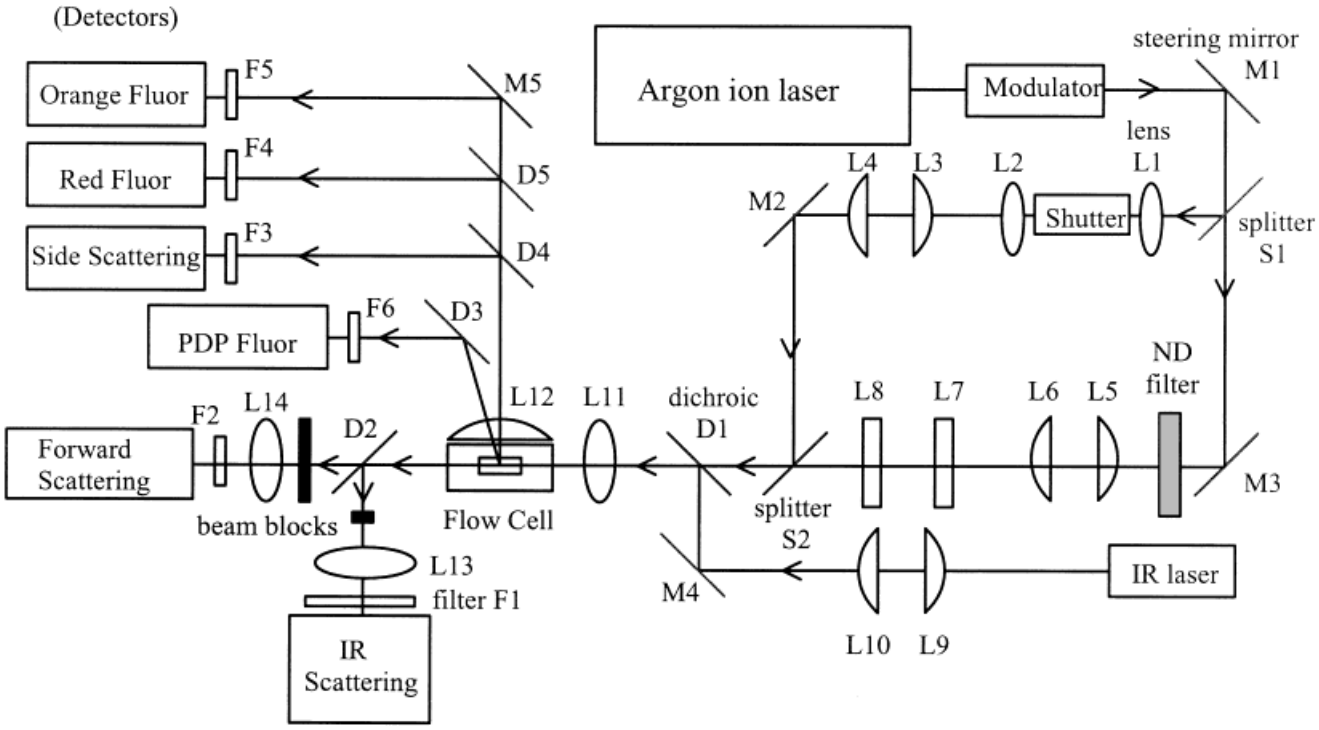


FIG. 2. Optical layout of the PDP flow cytometer. L, lens; M, mirror; D, dichroic; S, splitter; F, filter. The laser beam positions within the flow cell are reflected in the timing diagram in Figure 3A.

eter (Fig. 2). An adjustable miniature gear pump (Model 188 Micropump, Vancouver, WA) provides sheath flow (typically 5 ml min^{-1}), and a syringe pump (Model 22, Harvard Apparatus, South Natick, MA) introduces sample through the injection needle. The sample syringe and tubing to the flow cell are water jacketed to provide temperature control. Sample particles typically travel at 1 m s^{-1} in the flow cell and pass first through an IR diode laser beam (50 mW, 785 nm, Lasiris, Inc., Saint-Laurent, Quebec) which forms the basis for a cell detector (see below). Particles then pass through two beams derived from an air-cooled Ar ion laser (100 mW, 488 nm, Omnichrome, Chino, CA): the first, a weak beam for PDP excitation and the second, a stronger beam for conventional flow cytometric measurements. Initially, the 488 nm beam passes through an electro-optic modulator (Model 350, Conoptic Inc., Danbury, CT), which is normally closed (extinction ratio = 300:1) and which has an opening time of 1 μs, and then through an 80:20 beam splitter (S1, Fig. 2). The weak (PDP) beam excites Chl fluorescence for induction measurements, which are initiated by opening the electro-optic modulator. The strong beam, for conventional flow cytometric cell classification, is directed to a mechanical shutter (opening time 50 μs; LS500F, NM Lasers, Sunnyvale, CA) which is also normally closed. A second beam splitter (S2) serves to ''recombine'' the classification and PDP beams so that they are nearly coaxial. Both 488 nm beams pass through a dichroic mirror (D1) that directs the IR beam to the flow cell. All three laser beams are focused on the flow cell by an achromatic lens (L11, focal length = 40 mm).

A pair of cylindrical lenses (L7 and L8) is used to increase the vertical divergence of the PDP beam, and thus the vertical dimension of the PDP laser spot in the flow cell, to obtain a wide region of the beam with nearly constant intensity (Fig. 3A). Translations of cylindrical lenses (L3–L6) allow independent adjustment of the horizontal sizes of the PDP and classification laser spots.

Fluorescence and side-scattered light are collected by the FACScan lens assembly (L12). Because the PDP and classification beams are spatially separated, it is possible to direct signals from the two beams to independent detectors by using a dichroic filter (D3) as a steering mirror. Light originating from the classification beam passes below this filter, while Chl fluorescence excited by the PDP beam is reflected into the PDP PMT (Hamamatsu R1477 with 530 long pass absorbance and 660–700 nm bandpass interference filters). Dichroic filters (D4, D5) and a mirror (M5) in the path of the classification signals direct side scattering, red fluorescence (660–700 nm), and orange fluorescence (550–590 nm) to PMT detectors. Forward scattered light from the IR and classification beams is split by a dichroic mirror (D2) and directed by two lenses (L13, L14) to an avalanche photodiode detector module (Hamamatsu C5460) and a PMT (Hamamatsu R1923 with integrated preamplifier), respectively.

The PDP flow cytometer electronics consist of the IR triggering channel, the PDP measuring channel, and con-

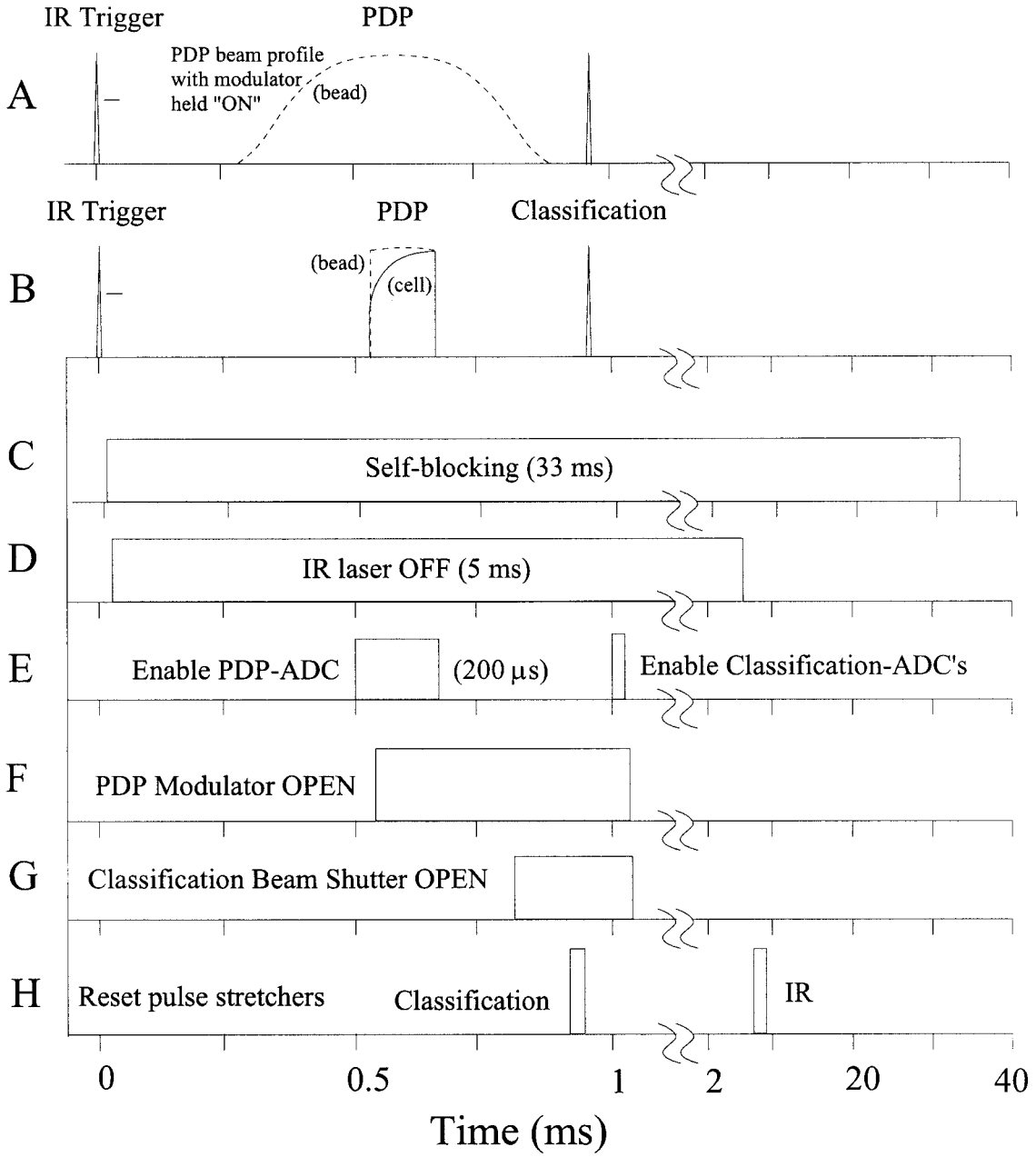


FIG. 3. Timing diagram for the PDP flow cytometer. Panels A and B represent schematic signals from the three laser beams, with the PDP laser modulator held open (to indicate the intensity profile of the PDP beam) and in normal operation, respectively. Panels C–H denote the timing of the control and signal processing functions. Each of the blocks in C–H denotes a signal from the delay generator; all are pre-programmed for different times after the initial trigger from the IR beam. **(A)** Scattered light from a particle passing through the IR beam triggers the system (time = 0). For clarity, the PDP signal is shown exaggerated in intensity relative to the other two signals. The shape of the unmodulated PDP beam profile (i.e., a signal from a bead with the modulator kept open) is indicated. **(B)** The signal shape generated by the passage of a healthy cell through the modulated PDP beam is indicated by the solid line; a bead produces a signal indicated by the dashed line. **(C)** The system is prevented from responding to new trigger signals during the period required for storing the data from the triggering particle (33 ms). **(D)** The IR laser is turned off after triggering, to reduce background during the subsequent measurements. **(E)** PDP signal acquisition (200 µs) begins as the cell approaches the measuring region. **(F)** After 50 µs of PDP background data has been acquired, the PDP modulator opens to start the fluorescence induction measurement. **(G)** The classification beam shutter (which has a minimum 800 µs delay) opens after the PDP measurement is completed. **(H)** The classification pulse stretchers are reset after the opening of the shutter, but before the cell passes through the strong beam. The IR pulse stretcher is reset after the IR laser has been turned back on for the next sample.

ventional flow cytometer channels for particle classification. Signals from both the IR and classification detectors are amplified logarithmically (Analog Modules 381, Longwood, FL) and integrated by a custom circuit; the peak pulses are then stretched (Analog Modules 611) and digitized using an 8-channel 100-KHz ADC in a 100-MHz Pentium PC. PDP signals are smoothed with a resistor-capacitor chain (time constant = 0.25 µs) and digitized

during the measurement by a 2-channel 60-MHz ADC (Gage Applied Sciences 6012, Montreal, Quebec).

The approach of a particle to the flow cell is sensed by a custom-built peak detector looking at the IR forward scattering signals. A peak detector is required to eliminate differences in timing between large and small particles approaching the Gaussian intensity profile of the IR beam. Upon detecting a particle, a voltage pulse is sent to the inputs of two 4-channel digital pulse generators (Quantum Composers 9300, Bozeman, MT), which control the subsequent timing of measurements and data acquisition (Fig. 3). The timing is adjusted so that when the particle is in the center of the flow cell, the PDP-ADC board is activated for 200 μs and the PDP beam modulator is opened (after a delay of 50 μs) to monitor Chl fluorescence induction over 150 μs. The photomultiplier signal is split and amplified on the board at two different gains to increase dynamic range; each signal is then digitized with 12-bit resolution at 5 MHz. When the PDP measurements are complete, the electromechanical shutter blocking the classification beam opens to allow conventional flow cytometric measurements of light scattering and fluorescence.

In addition to controlling the modulator and shutter openings and triggering data acquisition, the pulse generators reset the peak stretchers after each measuring cycle, provide self-blocking to avoid triggering by new cells during event processing, and turn off the IR laser to reduce background when measuring the PDP and classification responses.

The dark current from the PDP PMT (obtained by recording the fluorescence signal before the laser modulator was opened) is subtracted from the signals. Background contributed by the laser pulse (measured either by analyzing particles with no Chl fluorescence, or by decreasing the threshold on the IR scattering signal so that the system is triggered by noise in the absence of any particles) is also subtracted from each signal.

Because the PDP beam is a widened Gaussian rather than perfectly uniform, even cells traversing only its central portion do not experience an ideal rectangular pulse of light. We therefore monitor the shape of the excitation pulse by measuring the time dependence of fluorescence from red-fluorescing latex microspheres (1 μm diameter Transfluospheres, Molecular Probes) added to the sample. The signal profile of these beads, which have a constant fluorescent yield, is used to normalize the PDP induction curves. The intensity of the PDP laser beam is adjusted with neutral-density filters so that cell fluorescence saturates in 30–100 μs, and the rate of sample flow is adjusted to ensure that only one cell is in the PDP beam at a given time.

Data acquisition is controlled by a program developed using Visual Designer (Intelligent Instrumentation, Inc., Tucson, AZ) software running under Windows 3.11, allowing real-time control of the PDP- and classification-ADC boards, and storage of both the PDP induction curve and classification data for each cell. Typically we accumulate 1,000 to 25,000 events from each sample. Using software adapted from ''Cytowin'' (D. Vaulot, www.sb-roscoff.fr), subpopulations of cells (or beads added as internal standards) are delineated based on the flow cytometric signatures, and PDP induction curves comprising the summed data from each subpopulation are obtained. The PDP data are fitted to a biophysical model to estimate the photosynthetic parameters ϕ and σI using Matlab software (Mathworks, Inc.) (6). Because all the cells in a given sample should be exposed to the same I, differences in σ between populations can be inferred from the measurements.

RESULTS AND DISCUSSION

The PDP instruments were used to analyze natural populations of phytoplankton in water samples obtained from Niskin bottles, net tows, and on-deck incubation experiments in the Southern Ocean, as part of the US JGOFS Southern Ocean Process Study (''Process II'' cruises of the R/V *Nathaniel B. Palmer* and the R/V *Roger Revelle*). We present selected examples below to illustrate some of the capabilities of the instruments.

PDP Microfluorometer

The PDP microfluorometer provides the capability to measure phytoplankton at the species level, since each cell is visualized before measurement. Fluorescence induction curves can be obtained from individual cells as small as a few micrometers in diameter, such as the prymnesiophyte *Phaeocystis* spp. (5 μm diameter) (Fig. 4), which were encountered in natural samples and in iron enrichment incubation experiments. For much larger cells such as the centric diatom *Corethron* spp. (>100 μm), only a portion of the cell is measured (Fig. 5), but we found no differences in photosynthetic parameters when different parts of a single cell were measured. Signals from cells such as *Corethron* can be large enough to analyze from a single flash, rather than by averaging the results of many flashes. The fact that the fluorescence induction during the first flash is very similar to that of the average of 50 flashes gives us confidence that we are not changing the photosynthetic state of the cell significantly by repeated excitation. It also suggests that even smaller cells may be analyzed by utilizing photon counting and many repetitions, contrary to our earlier expectations (6), which were based on measurements at higher repetition rates and without temperature control.

We expect temperature control to be important for measurements of physiological state, especially for Southern Ocean cells adapted to temperatures far lower than those of the laboratory. The temperature control afforded by the water-jacketed microfluorometer flow cell was sufficient to maintain cells in good condition for extended periods, as indicated by the example of a dinoflagellate with $F_v/F_m = 0.6$, which was remeasured after 12 h of confinement, with no change in F_v/F_m (data not shown). When the water cooling was subsequently shut off, F_v/F_m declined to near zero within 20 min.

For *Phaeocystis*, *Corethron,* and *Fragilariopsis* spp., an increase in F_v/F_m was observed when cells were enriched with 1–4 nM iron, indicating that these species were

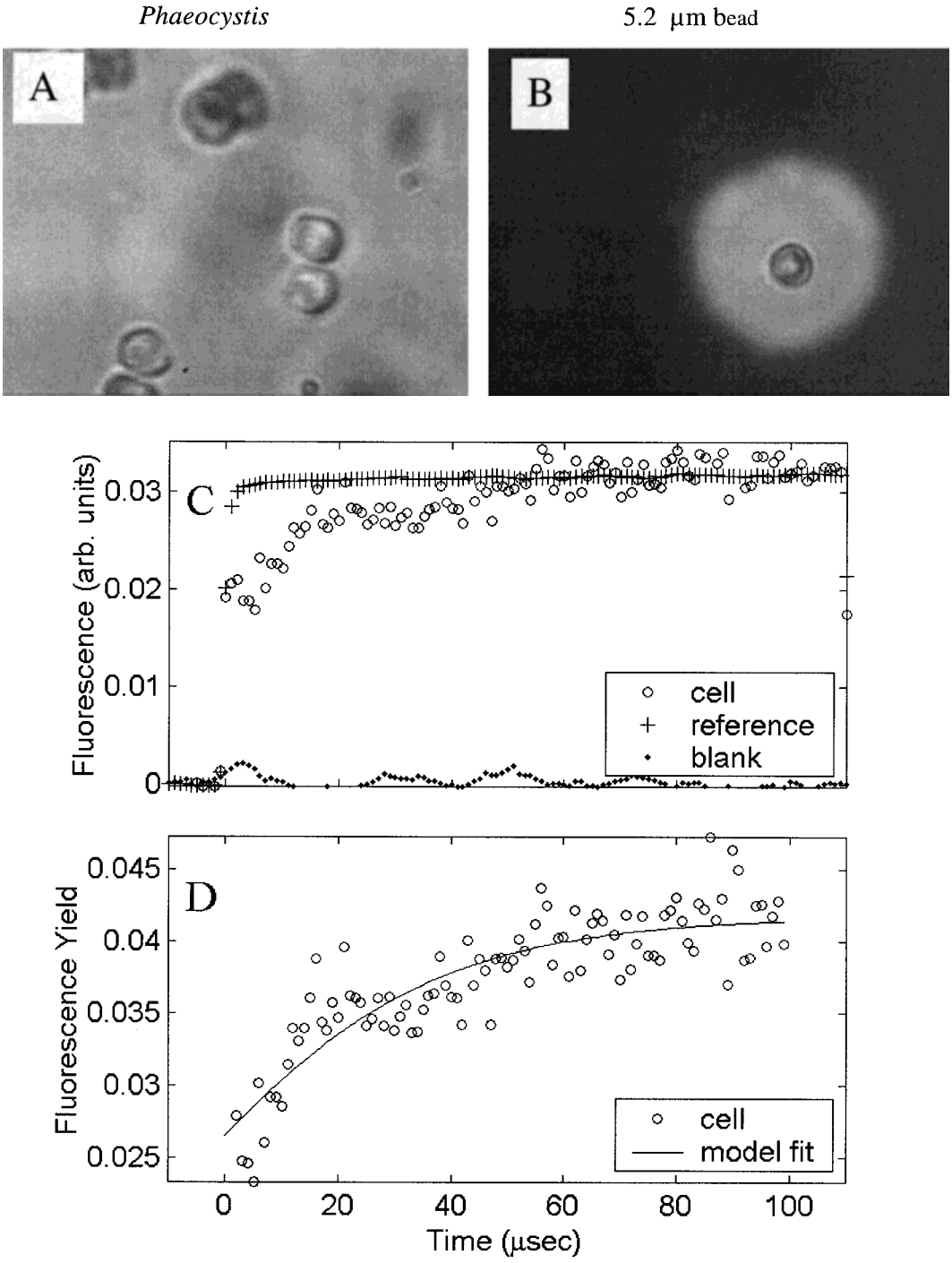
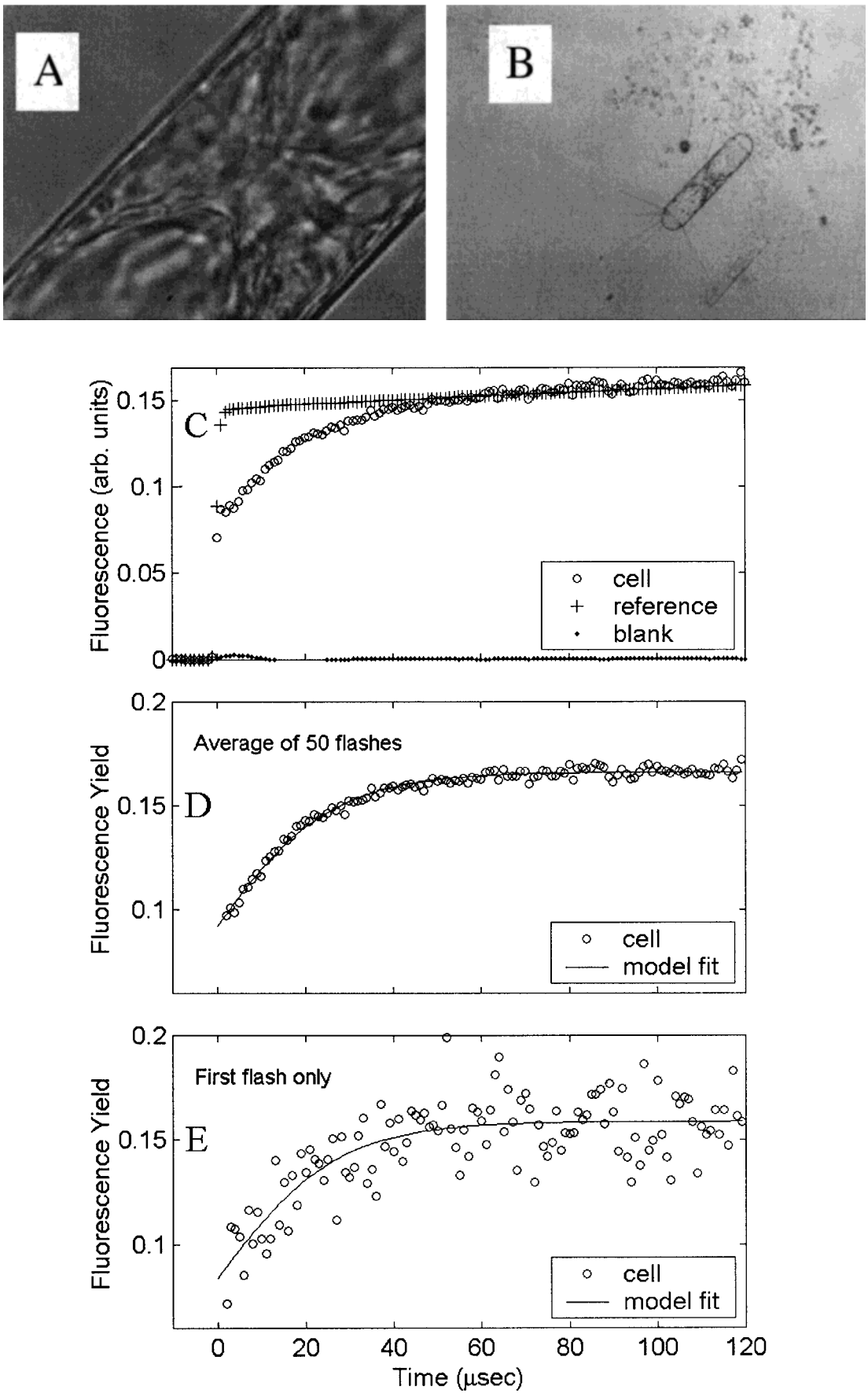


FIG. 4. PDP microfluorometric measurement of a pair of *Phaeocystis* cells collected in the Ross Sea, south of New Zealand. **(A)** Several cell pairs from a colony viewed with the imaging system under the measurement conditions. **(B)** The aperture used to isolate cells of interest for the measurement is shown at the same magnification as (A) with a 5.2 µm bead for scale. **(C)** Time dependence of fluorescence intensity for the cell pair, reference (excitation flash) and blank (empty field of view). **(D)** Time dependence of relative cell fluorescence yield. Least squares fit of the data to a biophysical model yielded estimates of $F_o = 0.0265$ and $F_m = 0.0445$ (relative to bead), for $F_v/F_m = 0.40$.

iron-depleted in situ (e.g., Fig. 6; Olson et al., in preparation). This finding is consistent with observations from in situ iron enrichment experiments in the equatorial Pacific (5,7), with laboratory experiments on iron-limited phytoplankton cultures (2,4), and with some previous work suggesting iron-limitation of Southern Ocean phytoplankton (8,9). In addition, we note that the distribution of relative variable fluorescence values within both control


Corethron (100X)
(10X)
A
B
C
Fluorescence (arb. units)
0.15
0.1
0.05
0
cell
reference
blank
D
Average of 50 flashes
Fluorescence Yield
0.2
0.15
0.1
cell
model fit
E
First flash only
Fluorescence Yield
0.2
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20
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100
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Time (μsec)

FIGURE 5.

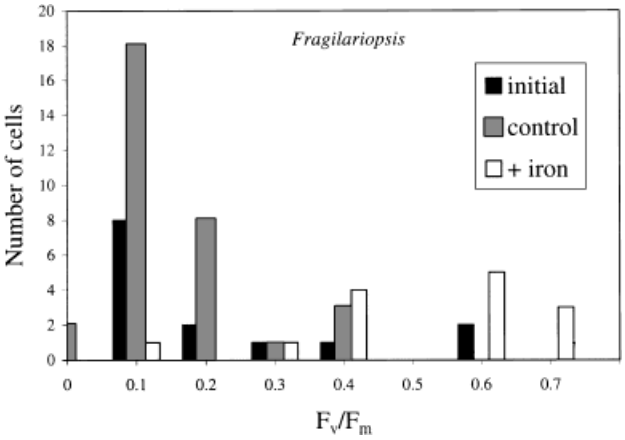

FIG. 6. Pump-during-probe microfluorometry results for *Fragilariopsis* spp. cells from the initial sample and from control- and iron-enriched bottles on day 5 of an iron-enrichment incubation experiment south of the Polar Front in the northern Ross Sea. While iron-treated cells exhibited the highest average F_v/F_m, the distribution of values was wide in each case; some cells in the initial sample were quite ''healthy'' even though the average F_v/F_m was low. Likewise, even in the iron-enriched sample, a few cells were in poor condition.

and iron-enriched populations was wide; some control cells had relatively high F_v/F_m while occasional cells in the enriched sample were in poor condition. With more measurements, such distributions may reveal valuable information about mechanisms of species succession in response to environmental forcing.

Microfluorometric measurements of the turnover time for PS2 reaction centers can be obtained by monitoring the decay of fluorescence yield after light saturation (Fig. 7). We have begun to explore this aspect of the measurements, but have not yet exploited it fully. Our present system is not optimized for measurements of the very short decay flashlets, which are necessary to minimize reclosing of reaction centers, and we have not yet considered the actinic effect of the flashlets in estimating reaction center turnover (although a more complex model could readily be applied). Even with these limitations, we note that for this cell the fast component of fluorescence decay (τ_1) was observed to be 150 μs, consistent with earlier estimates in phytoplankton cultures (3,4). The slow component of fluorescence decay (τ_2) was estimated to be 9 ms, which is much longer than the time scale of our measurements, and so would have little effect on the results.

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FIG. 5. PDP microfluorometric measurement of a *Corethron* cell. **(A)** The cell viewed with the imaging system under measurement conditions; a subset of this view was assayed using the aperture as shown in Fig. 4B. **(B)** The same field at 10-fold lower magnification reveals the entire *Corethron* cell as well as part of a *Phaeocystis* colony. **(C)** Time dependence of fluorescence intensity for the cell, reference (excitation flash) and blank (empty field of view), averaged over 50 flashes. **(D)** Time dependence of cell fluorescence yield, averaged over 50 flashes. Least squares fit of the data to a biophysical model yielded estimates of F_o = 0.092 and F_m = 0.175 (relative to bead), for F_v/F_m = 0.47. **(E)** Time dependence of cell fluorescence yield for the first flash in the measurement series. Though the single-flash data are noisier, the estimates of F_o (0.084), F_m (0.168) and F_v/F_m (0.50) were close to that for the averaged data above.

PDP Flow Cytometer

The PDP flow cytometer provides fluorescence yield induction curves linked with flow cytometric light scattering and fluorescence measurements for individual particles (Fig. 8). For natural samples from the Southern Ocean, fluorescent beads added as internal standards and cryptophytes (containing phycoerythrin) were distinguished by the presence of orange fluorescence in addition to red fluorescence, and pennate diatoms were distinguished from other cells by their characteristically small forward light scattering relative to red fluorescence (10). The main population of phytoplankton cells was subdivided into ''small'' (about 2–5 μm diameter) and ''large'' cells according to their forward light scattering signals. PDP fluorescence induction measurements were made on the individual cells prior to flow cytometric classification, and the data averaged for each subpopulation. To obtain relative fluorescence yield, cell PDP fluorescence was normalized to that of the 1-μm red-fluorescing plastic beads (Fig. 8C). The results suggest that the only cell group in good physiological condition, with F_v/F_m near the ''maximum'' value of 0.65, was the cryptophytes. The other cell groups were in relatively poor physiological state, with F_v/F_m values of only about 0.3. In addition, the functional absorption cross section for PS2 was much lower for the cryptophytes than for the other cells. Although the differences between groups in both relative variable fluorescence and functional absorption cross section are consistent with iron limitation of non-cryptophyte cells (2,4), the latter difference could also reflect the relatively weak absorption of the 488-nm excitation light by the cryptophytes' major pigment, phycoerythrin. The product σI for the other cell groups decreased with increasing cell size, which is consistent with the effect of pigment packaging in different-sized cells (11). In a cell with a high package effect, we expect some RCs to saturate more slowly due to reduced excitation intensity from intracellular shading.

While it was generally necessary to average PDP fluorescence measurements from many cells in a population to obtain reliable induction curves, single cell measurements can be practical with the flow cytometer. For certain cell types, such as the largest, most pigmented cells encountered near the Polar Front, good quality induction curves were observed for single cells (Fig. 8E, F), and it may be possible to examine cell-to-cell variability in such populations.

Comparison to FRR

Estimates of F_v/F_m obtained by averaging all the phytoplankton cells measured by the PDP flow cytometer were similar to estimates from a commercial FRR fluorometer (FastTracka, Chelsea Instruments) measuring bulk water samples (e.g., Fig. 9). This agreement is expected because the two instruments operate on the same principle: both

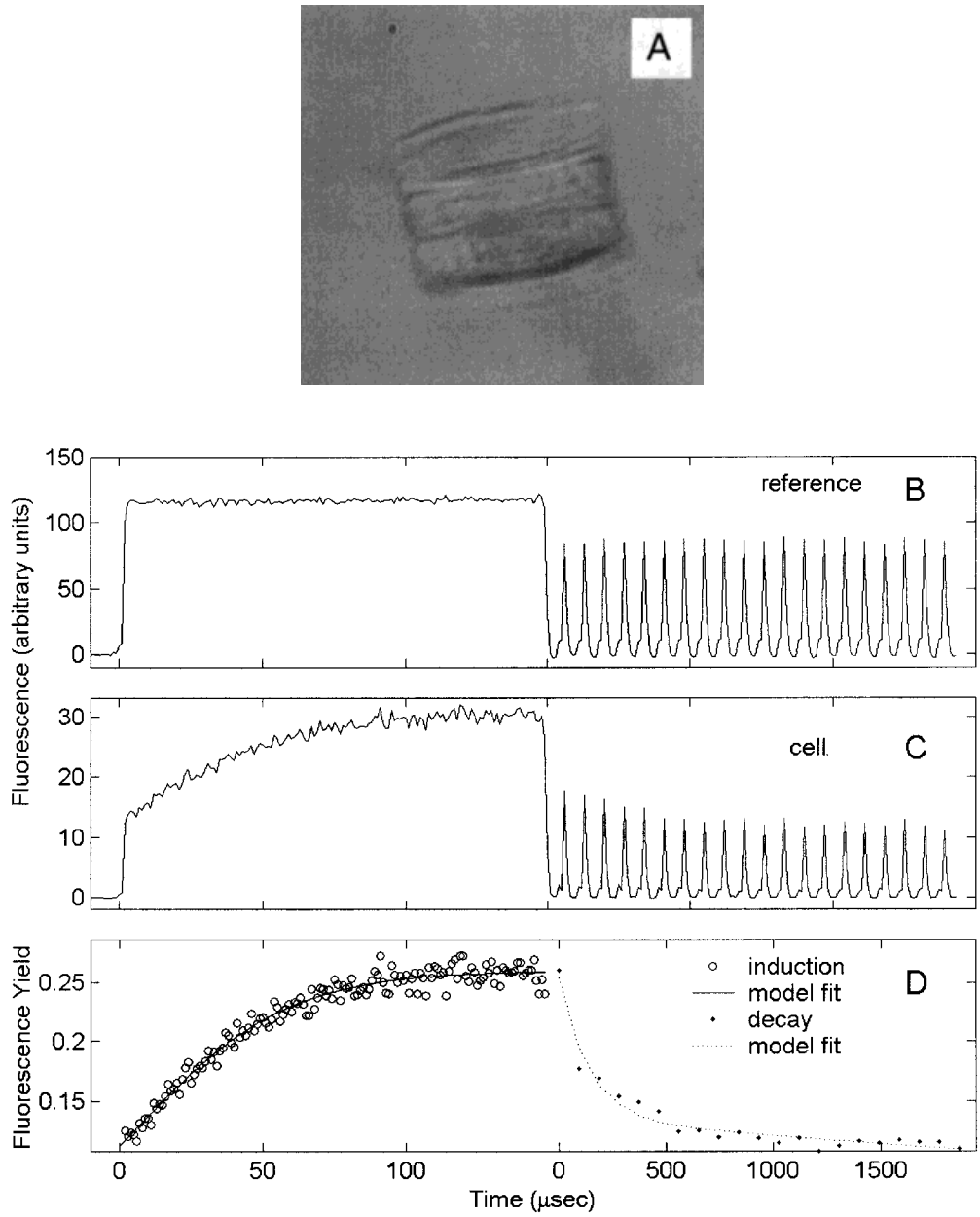

FIG. 7. Pump-during-probe microfluorimetry of *Fragilariopsis* spp. **(A)** from the iron-enriched bottle of Fig. 6, showing the post-saturation decay in fluorescence yield. **(B)** Reference signal from flash sequence. **(C)** Cell fluorescence. **(D)** Fluorescence yield. F_o was estimated at 0.112 and F_m at 0.309 (relative to bead), for $F_v/F_m = 0.64$. Note in (D) the change in scaling of the time axis between the saturation and decay phases. In (B) and (C) the decay phase signals are shown with the same time resolution as for the saturation phase data, but the 100 μs intervals between flashlets are omitted.

instruments measure fluorescence induction on the time scale of a single turnover of reaction centers. In the FRR, the rate constant for re-opening (τ) is measured for each sample. Measurement of τ is not feasible in the flow cytometer due to the short time each cell is in the measurement region, so it is important to minimize the effect of turnover by achieving saturation quickly relative to turnover time. The other difference between the two instruments, the supply of excitation as flashlets by the FRR versus continuously by the flow cytometer, should not affect the final result.

Limitations, Sea Going Operation, and Future Directions

Flow cytometric estimates of photosynthetic parameters are derived from single cell measurements, but for most cells the signals are very small, so that population averaging is necessary to achieve an acceptable signal-to-

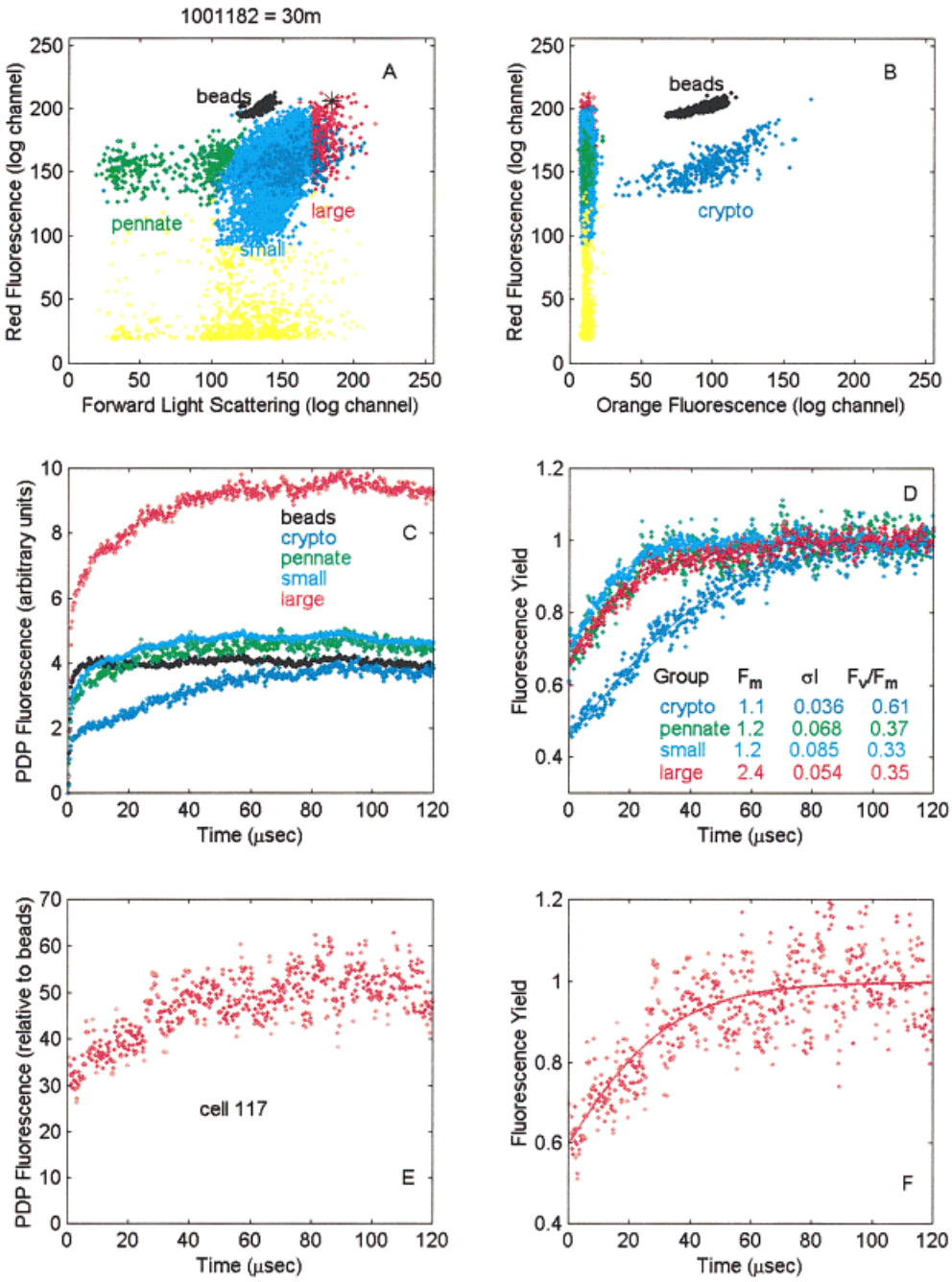

FIG. 8. PDP flow cytometric analysis of the phytoplankton in a water sample from 30 m depth south of the Polar Front in the northern Ross Sea. Each dot in panels **A** and **B** represents a particle with the indicated light scattering and fluorescence values. Color coding indicates categories of cells selected through multiparameter analysis. The fluorescence data for each group selected was averaged **(C)** and normalized to that of the beads to give fluorescence yield **(D)**. The yields in (D) have been scaled to F_m to facilitate comparisons of curves between populations. The number of particles in each group were: beads = 1852, pennate diatoms =359, cryptophytes =332 , ''small phytoplankton'' = 6493, and ''large phytoplankton'' = 438. Individual cell fluorescence induction curves from the largest cells measured (such as the one indicated by the asterisk in (A) had reasonable signal-to-noise ratios **(E)**; F_v/F_m for this cell was estimated at 0.44 **(F)**.

noise ratio. While population level information is very useful, for some ecological questions cell-specific information is necessary. For large highly pigmented cells, we have found that photosynthetic parameters can be successfully estimated from single-cell induction curves (Fig. 8F). This suggests that information about distributions of properties among individual cells can be obtained with the PDP flow cytometer for some groups of phytoplankton. Im-

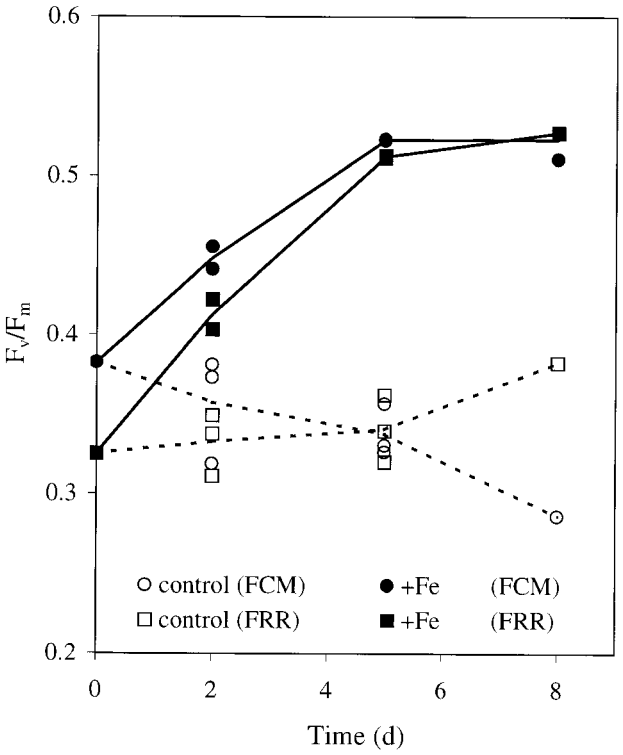

FIG. 9. Comparison of bulk (FRR, squares) and single-cell (PDP flow cytometer, circles) measurements of relative variable fluorescence yield (F_v/F_m) during an iron enrichment incubation experiment south of the Polar Front in the northern Ross Sea. Iron-enriched (filled symbols) and control (open symbols) bottles were sacrificed at each time point and measurements made within 1 h; results for replicate bottles for the 2- and 5-day time points are indicated. For this ''bulk'' comparison, all the cells in the flow cytometer signature were considered as a single population; both approaches indicate an increase in the F_v/F_m of the total phytoplankton population after iron enrichment.

proved identification of these cells is needed, however, for this approach to be most useful; such identification might be achieved by use of fluorescent antibodies or other taxonomic probes, or from an imaging-in-flow system (12,13). Improved characterization is also desirable to identify cells whose length approaches the vertical dimension of the PDP beam (150 μm), such as chains of diatoms, since their measurement should not be considered reliable.

The present system of analog PDP signal amplification cannot be used for cells smaller than about 2 μm. This did not pose a serious limitation for our initial work in the Southern Ocean where the phytoplankton are dominated by larger cells, but it would limit the usefulness of the instrument in warmer oceans where picoplankton are important. We know from previous work that even the smallest picophytoplankton, *Prochlorococcus* spp., can be assayed by PDP flow cytometry by utilizing photon counting (6), and we plan to implement photon counting on the sea going instrument. For this application, we will return to the use of a water-cooled Ar ion laser (6) rather than the present air-cooled model (which, though convenient, has significantly higher electronic and optical noise levels).

The major limitation on the use of the PDP flow cytometer at sea has proved to be ship motion. In even moderate seas, changes in acceleration due to pitch and roll caused the velocity of the sample through the flow cell to change noticeably in the laboratory version of the instrument. The most obvious effect of velocity changes is that cells no longer are all exposed to the same region of the PDP excitation beam; to the extent that the beam is not flat, this causes artifacts in the population-level induction curve. This problem was alleviated to a large extent by having the sheath intake and outflow points at the same height (so that the head pressure is constant), and by preventing movement of the tubing carrying sheath and sample fluid. A constant head system for the sheath intake was also used to prevent changes in pressure. These precautions allow operation in conditions of up to about 10° ship rolls. In more severe sea states, alignment of the laser beam through all the components of the optical path was affected, requiring frequent adjustment. These problems could be alleviated by further development. For example, many of the optical components of the system could be eliminated if fiber optics and diode lasers (which can be switched on and off on microsecond time scales) were used for the PDP and classification beams. These refinements can be pursued if the results of analyses with the present instruments warrant.

Both the flow cytometer and microfluorometer were mounted on pneumatic vibration dampers on board ship (which is standard technique for microscopy at sea), but we cannot say how necessary this precaution was since we never operated them without vibration damping. The microfluorometer was much less affected by ship motion than the flow cytometer, because the sample was immobilized in a closed chamber during analysis. Flexure of the microscope body during heavy ship motion was minimized by constructing a support framework around the microscope body. Although the measurements themselves require close to 1 min per cell, the major limitation of the microfluorometer measurements was the time required to locate subject cells, which entails gently concentrating a sample and manually loading and scanning the chamber. It is possible that some of this time could be saved by implementing an automated image detection system.

CONCLUSIONS

Results of initial field applications of the pump-during-probe microfluorometer and flow cytometer demonstrate that photosynthetic parameters can be obtained from measurements of individual phytoplankton cells in natural water samples. Single cells of sizes >5 μm can be assayed with the microfluorometer, and populations of cells with the flow cytometer. This approach should be a powerful tool for investigating the regulation of phytoplankton growth, and for estimating contributions of different groups of phytoplankton to primary production in the sea.

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