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Determination of photosynthetic pigment composition in an individual phytoplankton cell in seas and lakes using fluorescence microscopy; properties of the fluorescence emitted from picophytoplankton cells

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Abstract

To develop a method for the determination of photosynthetic pigment species in individual phytoplankton cells, especially natural picophytoplankton cells, the fluorescence spectra of intact cells were studied with cultured phytoplankton. The study was made mainly with phycoerythrin-containing picophytoplankton collected off Japan in 1982 with reference to diatomal species and phycoerythrin-free cyanophycean species. The spectra were measured for cell suspensions with an ordinary spectrofluorometer, and for individual cells with a microscope spectrofluorometer, paying special attention to the effect of cell-fixation. Results indicated that: (1) the cell-fixation with the glutaraldehyde and paraformaldehyde mixture modified phycoerythrin emission from picophytoplankton markedly in its wavelength location and intensity, but (2) the emission from phycocyanin was affected far less, and (3) the emission from chlorophyll *a* was not altered. However, the phycoerythrin emission modified by the fixation was found to be easily distinguished from other emissions, and kept its intensity high enough for detection with a fluorescence microscope. The fluorescence properties after the fixation were kept unaltered for a long period of time. Based on the results, we propose a simple method for the determination of photosynthetic pigments in individual phytoplankton cells in seas and lakes using fluorescence microscopy. Results of our tests with natural samples of phytoplankton are presented, and problems for further improvement are also discussed.

Introduction

Recent studies have revealed a wide occurrence of unicellular picophytoplankton in oligotrophic ocean environments (Fournier, 1970; Manton, 1977; Johnson and Sieburth, 1979, 1982; Waterbury *et al.*, 1979; Krempin and Sullivan, 1981; Li *et al.*, 1983; Platt *et al.*, 1983; Takahashi and Hori, 1984). Among the picophytoplankton, the cyanophycean species seems to be the most abundant (Johnson and Sieburth, 1979; Krempin and Sullivan, 1981). However, a green algal species has also been found (Fournier, 1970; Manton, 1977; Johnson and Sieburth, 1979; Takahashi and Hori, 1984), suggesting that this plankton group does not consist of a single phylum. Its abundance in oligotrophic sea areas indicates that it is important as the primary producer in such sea areas. The species structure of this plankton group is a primary requirement for the understanding of the primary production in such sea areas.

Since picophytoplankton are so small, species identification is difficult to determine by means of morphological characters under a light microscope. Thus far it has been reported that cyanophycean species have phycoerythrin (PE) as their main light-harvesting pigment for photosynthesis, so that the emission from this pigment can be used to characterize most cyanophycean species. Emission occurs in the orange region (580 nm) of visible light, differing from the far-red chlorophyll *a* (Chl *a*) emission (685 nm). Such a difference has been used to discriminate PE-containing cells from those of other phyla using a fluorescence microscope (Johnson and Sieburth, 1979; Waterbury *et al.*, 1979).

However, the picoplankton population is not always large enough for a direct cell-counting of water samples, and cell-counting often cannot be made immediately after sampling. Such a delay may cause a reduction of the emission yield due to the breakage of cells or decomposition of the pigment itself within cells. Tsuji and Yanagita (1981) reported that cell-fixation with glutaraldehyde and paraformaldehyde is mild enough to preserve fragile phytoplankton cells for a long period of time. This method is sufficiently mild to allow the preservation of the picophytoplankton sample for fluorescence counting.

We made an experiment to determine the effect of this fixing treatment on the emission from PE-containing picophytoplankton and supplementarily from species of

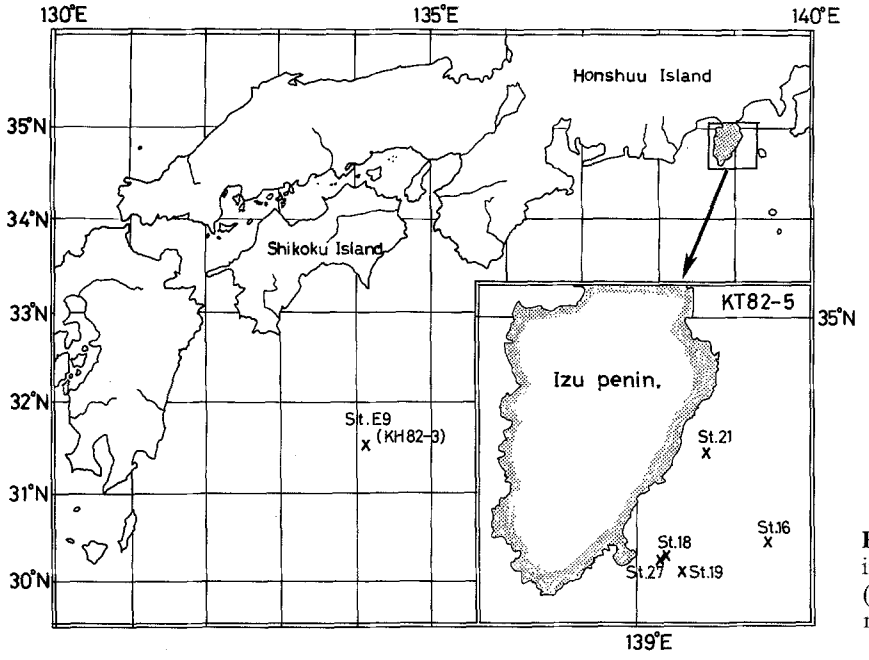

Fig. 1. Stations for collecting natural samples during the research cruises of R/V "Tansei-maru" (KT82-5, May 1982) and of R/V "Hakuhoh-maru" (KH82-3, St.E9, June 1982)

different pigment compositions. The experiments were made for cultured phytoplankton and for natural samples. The results reported here indicate that the most extensive modification occurs in the PE emission in its wavelength location and its intensity, but that it is still clearly distinguishable from other emissions. A method for the counting of phytoplankton in natural samples with the combined use of fluorescence microscopy and glutaraldehyde-paraformaldehyde fixation is also proposed.

Materials and methods

Algal cultures

Picophytoplankton strains, *Synechococcus* sp. NIBB1024, isolated from the coastal water off Port Aransas, Texas, USA and *Synechococcus* sp. NIBB1025, 1026 and 1070, isolated from the Kuroshio waters off the Izu Peninsula, Japan, were used. The diatomal strains *Skeletonema* sp. NIBB3012, isolated from the Kuroshio water off Izu Peninsula was also used. They were grown at 25 °C in F-medium of Guillard and Ryther (1962) enriched for $NaNO_3$ at 500 mg l^{-1} and Na_2HPO_4 at 50 mg l^{-1}. A PE-free cyanophycean species, *Anacystis nidulans* (M1, Algal Collection of Institute for Applied Microbiology, University of Tokyo), was grown in MDM (Watanabe, 1960) at 30 °C. *Dunaliella tertiolecta* (Provasoli strain) was grown in CS medium (Blankley, 1971) at 20 °C. Light sources were incandescent light (35 W m^{-2}) for *Synechococcus* sp. NIBB1024, 1025 and 1026 and *A. nidulans,* and fluorescent light (Toshiba, white-type, 20 W m^{-2}) for *Skeletonema* sp. and *Synechococcus* sp. NIBB1070.

Fixation of cultured algal cells

Cells were fixed with a mixture of glutaraldehyde and paraformaldehyde (Tsuji and Yanagita, 1981) in culture medium. After being left for 1 h at room temperature (ca 22 °C), the fixed cells were further incubated at 4 °C for 40 h. For a long-time storage period, the fixed cells were collected by centrifugation, resuspended in glycerin, and kept at −20 °C (cf. Tsuji and Yanagita, 1981).

Natural picophytoplankton samples

Picophytoplankton samples were collected from waters of 0 to 125 m in depth from two Kuroshio regions around Japan (Fig. 1): off the Izu Peninsula during the research cruise of the R/V "Tansei-maru" (Ocean Research Institute, University of Tokyo, KT82-5 cruise, 1982), and off Shikoku Island during the research cruise of the R/V "Hakuhoh-maru" (Ocean Research Institute University of Tokyo, KH82-3 cruise, St.E9, 1982).

Immediately after sampling with a Van Dorn sampler, the water sample was fixed with the fixative, and filtered onto a membrane filter by gentle suction to prepare it for microscopy (Tsuji and Yanagita, 1981). The preparation was stored at −20 °C until microscopic observations could be made.

Measurements of fluorescence and absorption spectra of cell suspensions

Fluorescence spectra of the cultured cell suspensions were measured by a Hitachi MPF4 spectrofluorometer. The spectra were corrected for wavelength-dependent sensitivity

Table 1. Types of pigment composition in algal strains used for the fluorescence experiments

Types		Algal strains	Pigment composition
Type I:	C-PE containing species	*Synechococcus* sp. NIBB1024	C-PE, PC, APC, Chl *a*
		Synechococcus sp. NIBB1025	C-PE, PC, APC, Chl *a*
		Synechococcus sp. NIBB1026	C-PE, PC, APC, Chl *a*
Type II:	S-PE containing species	*Synechococcus* sp. NIBB1070	S-PE, PC, APC, Chl *a*
Type III:	PE-free species	*Anacystis nidulans* IAM-M6	PC, APC, Chl *a*
Type IV:	Phycobilin-free species	*Skeletonema* sp. NIBB3012	Fucoxanthin, Chl *c*, Chl *a*
		Dunaliella tertiolecta	Chl *b*, Chl *a*

Abbreviations: APC, allophycocyanin; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Chl *c*, chlorophyll *c*; PC, phycocyanin; PE, phycoerythrin
For details, see text

of the measuring system. Absorption spectra were measured by a Hitachi 340 spectrophotometer by the split beam method.

Measurements of fluorescence spectra with individual cells

The spectra of fluorescence from individual cells were measured by a microscopic spectrofluorometer (Karl Zeiss MPM 03FL) in an epifluorescence mode. The spectra were corrected for wavelength-dependent sensitivity of the measuring system. The amplification of the object lens was 100. The light source for excitation was a high pressure mercury lamp (100 W).

For PE and phycocyanin (PC) excitations, the 546-nm line was isolated by a combined use of a dichroic mirror (FT580) and a band pass filter (BP546). The fluorescence spectrum longer than 550 nm was measured (wavelength resolution, 10 nm half band-width); the excitation stray was removed by a combination of a dichroic mirror (FT580) and a sharp cut filter (Fuji, SC56). For Chl *a* excitation, the 436-nm line was isolated by a combined use of a dichroic mirror (FT460) and a band pass filter (BP436). The fluorescence longer than 470 nm was measured; the excitation stray was cut by a dichroic mirror (FT460) and a sharp cut filter (LP470).

Results

Fluorescence spectra of various algal species with and without fixation

Effect of the cell-fixation was examined with algal species with four different types of photosynthetic pigment composition: (I) species containing C-PE as the major phycobilin; (II) species containing special PE (S-PE)[1] as the major phycobilin; (III) species containing PC as the major phycobilin; and (IV) species which does not contain phycobilins. Algal species of the four types used for the experiments are presented in Table 1.

Type I: The absorption spectrum of the cell suspensions of *Synechococcus* sp. NIBB1024 is presented (Fig. 2A, curve a). Depending on growth conditions, the absorption peak at 565 nm due to C-PE was slightly changed in its strength relative to that of the 680-nm peak due to Chl *a*. A broad peak around 630 nm was due to PC. The pattern clearly indicated that PE was the main pigment in this case. The fluorescence spectrum (Fig. 2B, curve a) of the cell suspension corresponded to the pigment composition; the major emission at 580 nm was due to C-PE, and a secondary emission at 685 nm was due to Chl *a*. The emission due to PC (650 nm) was minor. The pattern measured by the microscope spectrofluorometer is also shown (Fig. 2C, curve a). Because of a low wavelength resolution (half band-width, 10 nm), the pattern became somewhat vague. This pattern was similar to the spectrum

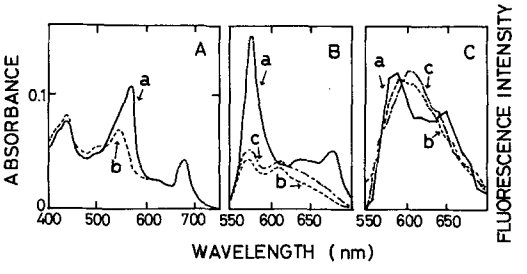

Fig. 2. *Synechococcus* sp. NIBB1024. Absorption and fluorescence spectra. In (A), absorption spectra of the cell suspension: curve a, before cell-fixation; curve b, 40 h after fixation. In (B), fluorescence spectra of cell suspension: curve a, before cell-fixation; curve b, 40 h after fixation; curve c, after storage for 74 d in glycerin at −20 °C. Cell concentrations were normalized to Chl *a* absorption at 680 nm. Excitation at 540 nm was made. In (C), fluorescence spectra of individual cells: curve a, before cell-fixation; curve b, 40 h after fixation; curve c, after storage for 74 d in glycerin at −20 °C. Excitation at 546 nm was made. For details, see text

[1] A special PE showing multi-absorption bands in visible region; cf. Alberte *et al.*, 1984; Ong *et al.*, 1984

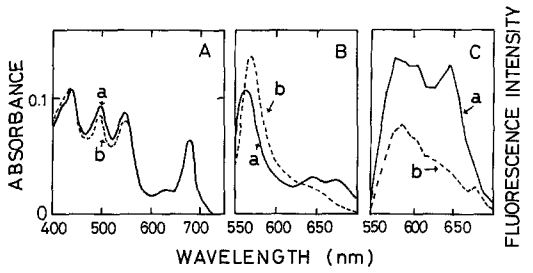

Fig. 3. *Synechococcus* sp. NIBB1070. Absorption and fluorescence spectra. In (A), absorption spectra of cell suspension: curve a, before cell fixation; curve b, 40 h after fixation; In (B), fluorescence spectra of cell suspension: curve a, before cell fixation; curve b, 40 h after fixation. Cell concentrations were normalized to the Chl *a* absorption at 680 nm. Excitation at 540 nm was made. In (C), fluorescence spectra of individual cells: curve a, before cell-fixation; curve b, 40 h after fixation. Excitation at 546 nm was made. For details see text

measured with the cell suspension except for the region of Chl *a* emission.

Cell-fixation by the method of Tsuji and Yanagita (1981) caused a significant modification of the absorption and fluorescence spectra (Fig. 2A, curve b; Fig. 2B, curve b). The absorption spectrum indicated that the modification mainly occurred in PE; reduction of the peak intensity was accompanied by a shift of the peak position from 570 to 540 nm. Changes in the fluorescence spectrum due to the pigment modification occurred as (1) a reduction of PE emission down to 1/2 to 1/4, and a shift to shorter wavelength (from 580 to 570 nm), (2) an appearance of a new emission at 610 nm, and (3) a disappearance of Chl *a* emission at 685 nm. These might be due to a modification of PE and to the uncoupling of energy transfer between PE through PC and APC to Chl *a*. Curve b in Fig. 2C shows the fluorescence spectrum of an individual fixed cell. The emission from PE has become weaker.

Despite such modification, the fluorescence from the fixed cell remained for a long period (74 d), when the cell had been stored at −20 °C after fixation. Curve c in Fig. 2B and curve c in Fig. 2C were obtained for cells stored for 74 d after fixation. Results indicated that the modification of the pigments due to cell-fixation was not progressive during storage, but it occurred only at the time of the fixation treatment. The same results were obtained for two other *Synechococcus* strains, NIBB1025 and NIBB1026 (data not shown).

Type II: Results obtained for the species (*Synechococcus* NIBB1070) containing S-PE are shown in Fig. 3. Cell-fixation did not cause any significant modification in the absorption spectrum (Fig. 3A, curve b). However, there was a modification of the fluorescence spectrum due to fixation (Fig. 3B, curve b). The extent of the modification of the fluorescence spectrum seemed to be less than that observed in Type I. This was not due to the character of this species, and was also variable in Type 1. The two examples illustrated in Figs. 2 and 3 represent extreme cases.

Types III and IV: Results for Types III (*Anacystis nidulans*) and IV (*Skeletonema* sp.) are shown in Fig. 4. In Type III (Fig. 4A), the main emission occurred from PC (650 nm), and *Chl a* emission (685 nm) was rather weak. The cell-fixation caused both a reduction of PC emission and an elimination of Chl *a* emission (Fig. 4A, curve b). A modification similar to that in Types I and II probably occurred in this case also. However, the modification of PC did not cause a shift of the emission peak toward shorter wavelengths, so that emission was insignificant at the wavelength region shorter than 600 nm where strong emission appeared in the case of PE-containing phytoplankton.

Only the Chl *a* emission was observable in the case of Type IV (Fig. 4B, curve a), and emission was not affected by the cell-fixation (Fig. 4B, curve b). Chl *a* emission was hardly observable under 546-nm excitation; the patterns in Fig. 4B were obtained under 436-nm excitation. The same results were obtained for the green algal strain *Dunaliella tertiolecta* (data not shown).

A photographic method for grouping natural a phytoplankton population by photosynthetic pigments

From the results described above, the emission patterns could be clearly distinguished from each other, depending on the pigment composition, even with the phytoplankton samples fixed and stored for 74 d. A sophisticated measurement by the microscope spectrofluorometer may be best for determining the pigments of the individual cells, although the measurement is time-consuming. As shown in Table 2, the excitation-emission characteristics are different in phytoplankton of different pigment types. This difference allowed us to develop a simple method for this purpose. A series of fluorescence microphotograms taken under three different conditions of excitation and emission can distinguish phytoplankton having at least three dif-

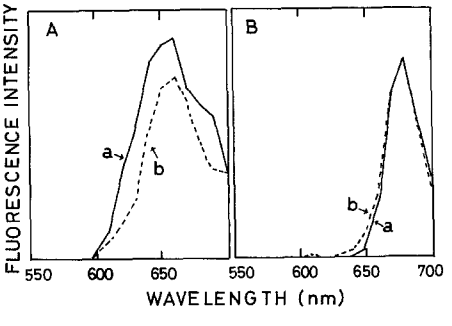

Fig. 4. *Anacystis nidulans* (A) and *Skeletonema* sp. NIBB3012 (B). Fluorescence spectra of individual cells. Curve a, before cell-fixation; curve b, 40 h after fixation. Excitations at 546 nm for *A. nidulans*, 436 nm for *Skeletonema* sp. were made, respectively. For details, see text

Table 2. Excitation-emission characteristics for fluorometric detection of phytoplankton cells with different pigment compositions

Algal type		Excitation		Emission peak		
		Blue (436 nm)	Green (546 nm)	Orange (500–600 nm)	Red (600–660 nm)	Far-red (680 nm)
Type I:	C-PE containing species	+	+	+ (–)	+ (–)	– (+)
Type II:	S-PE containing species	+	+	+ (–)	+ (–)	– (+)
Type III:	PE-free species	+	+	– (–)	+ (–)	– (+)
Type IV:	Phycobilin-free species	+	+	– –	– –	– +

Signs in parentheses mean possible results, but not determined in the present study. Type of pigment composition: See text and Table 1

ferent pigment compositions: for PE-species, with the coupling of the green excitation and the orange emission (550–600 nm), for PC-species, the coupling of the green excitation and the red emission (600–650 nm), and for phycobilin-free species, the coupling of the blue excitation and the far-red emission (680 nm).

Fig. 5 is an example of this trial made for the mixed sample with *Skeletonema* sp., *Anacystis nidulans* and *Synechococcus* sp. NIBB1024. The coupling of the 546-nm excitation and the emission longer than 590 nm showed the occurrence of both *Synechococcus* sp. and *A. nidulans* (Fig. 5 A). When the emission longer than 600 nm was eliminated by a band pass filter (BP580), only the occurrence of *Synechococcus* sp. was shown (Fig. 5 B). The phycobilin-free species *Skeletonema* sp. did not appear under green excitation. This species appeared in the photograms only with blue excitation (Fig. 5 C). The results clearly indicate the feasibility of this simple method.

Application to natural samples

To confirm the feasibility of our simple method in the natural environment, we applied the fluorescence microphotograph method to the samples obtained from Kuroshio waters (cf. Materials and methods). Phytoplankton populations in the samples determined by this method are presented in Table 3. In every case, PC-containing phytoplankton (Type III) were not detected. The method gave us the following information: (1) Picophytoplankton containing PE, probably the *Synechococcus* type, were abundant in these Kuroshio waters. (2) Picophytoplankton other than the *Synechococcus* type were very rare. Since the samples were obtained from different areas and depths and also at different times, the dominancy of PE-containing picophytoplankton suggests that the picophytoplankton of this type are an important primary producer in the Kuroshio waters around Japan.

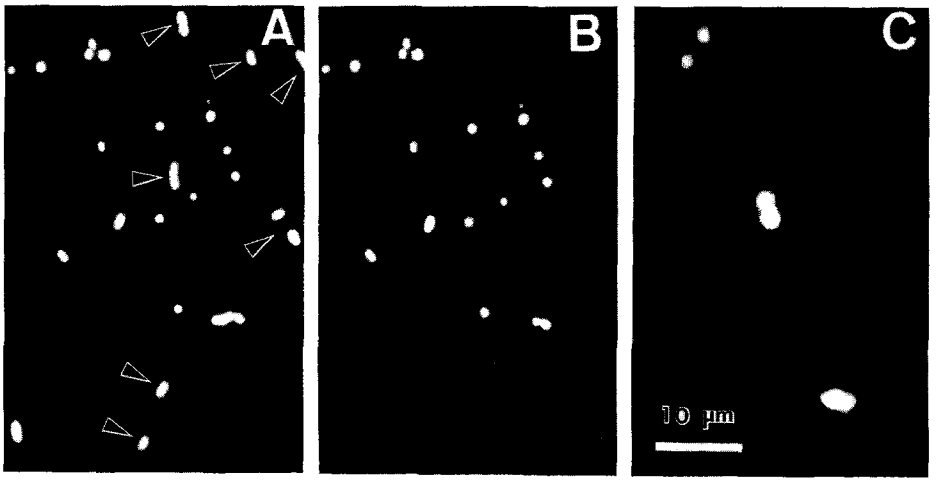

Fig. 5. *Synechococcus* sp. NIBB1024, *Anacystis nidulans* and *Skeletonema* sp. Epifluorescence microphotograms of the mixed population. Preparation for the microphotograms was made by the method of Tsuji and Yanagita (1981) without using glycerin jelly as the mountant. Combinations of excitation and emission were 546 nm and longer than 590 nm (A), 546 and 580 nm (B), and 436 nm and longer than 660 nm (C). The same microscopic field of the preparation was taken for the three photograms. Arrows in A indicate PC-containing *A. nidulans* cells which cannot be observed in B. For detail, see text

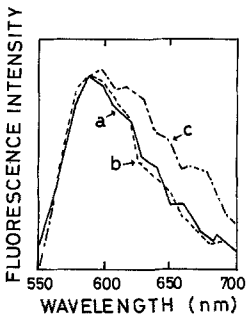

Fig. 6. Fluorescence spectra of individual cells of natural picophytoplankton. Picophytoplankton samples were obtained at St. E16 of the research cruise of R/V "Hakuhoh-maru" (July, 1982) (cf. Fig. 1). Samples from the waters at 50 m depth (curve a), 100 m depth (curve b) and 125 m depth (curve c) were fixed and stored at −20 °C by the method of Tsuji and Yanagita (1981). Excitation was made at 546 nm. For details, see text

According to Takahashi and Hori (1984), the green algal picophytoplankton occurred in the same sea areas. So far, however, we have failed to observe such picophytoplankton in our samples by our method. The difference could be due to the season (cf. Krempin and Sullivan, 1981) and the area of sampling. Since picophytoplankton of the *Synechococcus* type were also abundant in their case, the prediction from our determination is reasonable.

To confirm the above results, the fluorescence spectrum of individual cells from Kuroshio water was measured by the microscopic spectrofluorometer. The spectrum was measured for over 100 cells in each sample. Representatives are presented in Fig. 6. All spectra obtained were of the *Synechococcus* type (Fig. 2 vs Fig. 6). These results confirm that the photograph method can reasonably distinguish between the picophytoplankton of the *Synechococcus* type and other phytoplankton.

Discussion

The present method cannot distinguish a species containing C-PE from that containing S-PE. Because both types of PE show the emission at almost the same wavelength (Fig. 2C vs Fig. 3C), the difference among the types of PE cannot be detected by emission. The discrimination may be achieved by the measurement of absorption spectrum in the green region, or the absorption ratio at 500 vs 570 nm of an individual cell using the microscope spectrofluorometer in the absorption mode. A more feasible method may be the discrimination by a fluorescent antibody for the two PE types.

The discrimination of the species having Chl *a* and *c* from those having Chl *a* and *b* is also not possible with the present method. When we use excitation at the wavelength region for fucoxanthin and peridinin (500 to 530 nm) in addition to the Chl *a* excitation, the species with light-harvesting carotenoid can be detected by Chl *a* emission. Considering these possibilities, discrimination of phytoplankton species with different pigment compositions can be summarized in Table 4. For this purpose, a mercury lamp is not suitable as the light source. A xenon lamp provided with a suitable combination of filters should be better.

The excitation intensity of the microscope spectrofluorometer is generally extremely strong. Such a strong excitation often causes the photodestruction of the samples. In the present experiments, we observed a gradual decay of PE fluorescence during the measurement of the fluorescence spectrum by the microscope spectrofluorometer. A lack of Chl *a* emission in the measurements (Fig. 2B vs 2C or Fig. 3B vs 3C) may also be due to the photodestruction of energy transfer from phycobilin to Chl *a*.

Table 3. Occurrences of PE-containing and phycobilin-free phytoplankters in Kuroshio waters, off Japan

St.*	Depth	Phytoplankter** of Type I or II	Phytoplankter of Type IV	Ratio
No.	(m)	Cells per ml (A)	Cells per ml (B)	Percentage [A/(A+B)×100]
KT82-5* Research Cruise				
16	75	211	0.07	100.0
16	100	32	0.03	100.0
18	30	16 789	2.16	100.0
18	75	3 793	1.68	100.0
19	30	12 579	1.05	99.9
27	50	3 034	1.88	99.9
KH82-3* Research Cruise				
E9	0	3 546	1.20	100.0
E9	10	7 091	0.65	100.0
E9	30	3 191	3.10	99.9
E9	50	6 382	1.55	100.0
E9	75	1 418	0.70	100.0
E9	100	473	0.23	100.0
E9	150	236	0.03	100.0

* See Fig. 1
** Almost all were picophytoplankton

Table 4. Excitation and detection of the fluorescence from phytoplankton cells with different pigment compositions

Algal type	Excitation			Emission		
	Blue (435 nm)	Blue-green (500–530)	Green (540)	Orange (550–600)	Red (600–660)	Far-red (680)
Cyanophycean type (Phycobilin/Chl *a*)						
I. PE-major	+			−	−	+
		+		±*	±*	−
			+	+	+	−
II. PC-major	+			−	−	+
		+		−	−	−
			+	−	+	−
Chlorophycean-type (Chl *b*/*a*)	+			−	−	+
		+		−	−	(−)**
			+	−	−	−
Diatomal and dinoflagellate type (Fucoxanthin or peridinin/Chl *c*/*a*)	+			−	−	+
		+		−	−	+
			+	−	−	−

* Fluorescence may be weak
** Even when fluorescence occurs, the intensity is found to be very weak

A flash excitation source would be effective in reducing the excitation dose.

Another problem to be resolved is cell-fixation. The fixative used in this experiment contained paraformaldehyde, and the fixation was made at room temperature (ca. 22 °C). This may be too drastic to keep the pigment system in an intact state, though it is mild enough to keep the cell structure of fragile phytoplankton intact.

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