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Original Articles

# Cryptomonad Algal Phycobiliproteins as Fluorochromes for Extracellular and Intracellular Antigen Detection by Flow Cytometry

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**Background:** Phycobiliproteins play an important role in fluorescent labeling, particularly for flow cytometry. The spectral properties of R-phycoerythrin (R-PE) and allophycocyanin (APC) have made them the dominant reagents in this class of fluorochromes. In this study, we evaluate a lesser-known but potentially important series of low-molecular weight cryptomonad-derived phycobiliproteins (commercially termed the CryptoFluor™ dyes) for their applicability to flow cytometry, both in extracellular and intracellular labeling applications.

**Methods:** Several cell lines were labeled with biotin-conjugated antibodies against expressed extracellular surface proteins, followed by streptavidin conjugates of three cryptomonad phycobiliproteins (CryptoFluor-2, CryptoFluor-4, and CryptoFluor-5). Cells were then analyzed by flow cytometry using a variety of laser lines and emission filters to establish the optimal excitation/emission characteristics for each fluorochrome. Some cells were permeabilized and labeled for intracellular antigens, also using the cryptomonad fluorochromes. Where appropriate, parallel samples were labeled with other fluorochromes (including R-PE, APC, the cyanin dyes Cy3 and Cy5, and others) to gauge the performance of the cryptomonad fluorochromes against fluorescent labels previously evaluated for flow cytometry.

**Results:** CryptoFluor-2 possessed excitation/emission maxima similar to those of APC and Cy5, with good excitation in the red (HeNe laser 632 nm) and strong emission in the far red (660 nm). CryptoFluor-4 possessed excitation/emission maxima similar to those of Cy3, with optimal excitation in the green (Kr 530 nm) and strong emission in the yellow/orange (585 nm). CryptoFluor-5 possessed excitation/emission maxima similar to those of lissamine rhodamine, with optimal excitation in the yellow (Kr 568 nm) and emission in the orange (610 nm). All cryptomonad fluorochromes gave satisfactory results for both intracellular and extracellular labeling, with detection sensitivities that were comparable or better than traditional phycobiliproteins and low- molecular weight synthetic fluorochromes such as the cyanin dyes.

**Conclusions:** The CryptoFluor fluorochromes were applicable to flow cytometric immunodetection, with excitation and emission conditions commonly found on multilaser instruments. Performance of several of these dyes was at least comparable to existing fluorescent labels. The low molecular weights (30–60 kd) of phycobiliproteins may make them particularly useful in intracellular antigen detection. Cytometry 44:16–23, 2001.

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**Key terms:** flow cytometry; cryptomonad algae; phycobiliprotein; phycocyanin; CryptoFluor™

Phycobiliproteins are photosynthetic energy transfer proteins that possess distinctively strong pigmentation and unique fluorescent properties (1). Phycobiliproteins have been widely utilized as protein-conjugatable markers in fluorescence-based detection systems, particularly in flow cytometric immunophenotyping (2–4). They are water soluble, possess high-fluorescence quantum yields, have large Stokes shifts, and a resistance to photobleaching. These characteristics make them ideal candidates for fluorescence detection (1). In many cases, their excitation and emission maxima correspond to the laser emission



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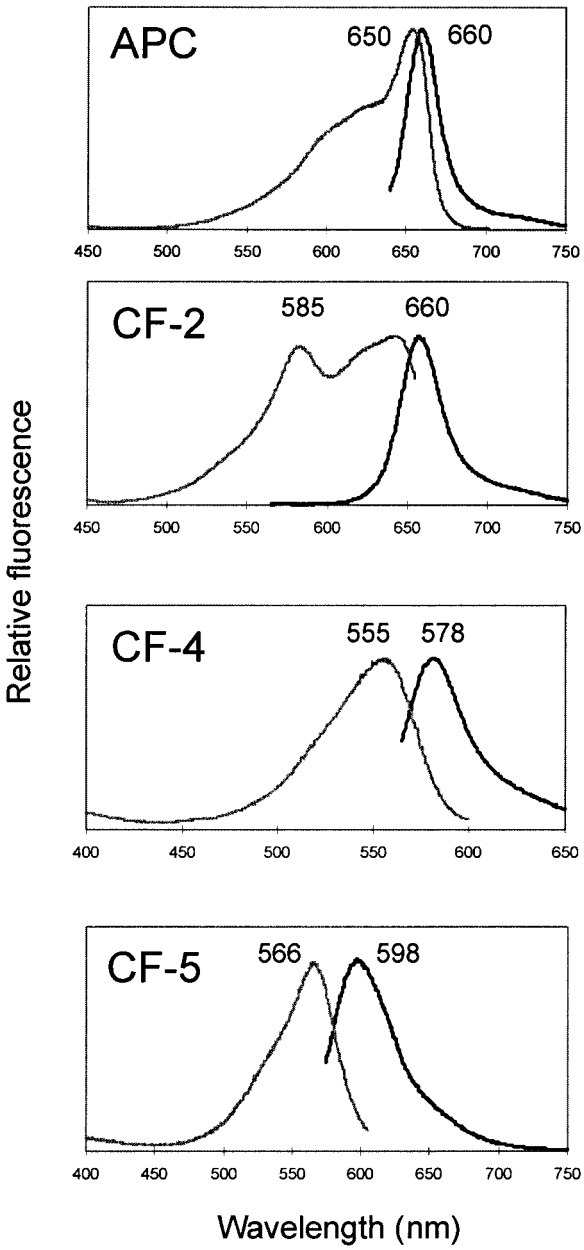


FIG. 1. Excitation/emission spectra of APC, CF-2, CF-4, and CF-5. Excitation curves are in grey, emission curves in black. Peak excitation/emission values for each fluorochrome are indicated. Excitation and emission curves were derived at the indicated excitation/emission peak values and are normalized for comparison purposes.

wavelengths found on many commercial flow cytometers. They are also capable of being linked to other dyes to produce tandem dyes at various emission wavelengths, extending their utility in multicolor detection. To date, the most widely utilized phycobiliproteins in flow cytometry are the R-form of phycoerythrin (R-PE, derived from certain red algal species) and allophycocyanin (APC, derived from cyanobacteria and red algae; 2,5). R-PE possesses fluorescence excitation maxima at 498 and 565 nm and an emission maximum at 573 nm; the excitation peak at 498 nm makes it ideal for flow cytometric detection using a standard argon-ion laser emitting at 488 nm. APC absorbs well from 610 to 650 nm with a maximum at 650 nm and an emission maximum at 657 nm. It can be excited efficiently by using dye head lasers emitting at 594 or 612 nm or HeNe lasers emitting at 632 nm. The emission ranges of both R-PE and APC are sufficiently separated from other commonly used fluorochromes (such as fluorescein) to have made them widely utilized in multicolor flow cytometry (6).

Despite the wide usage of R-PE and APC, there exist dozens of known phycobiliproteins from other eubacterial and algal species that may function well as fluorescent markers for flow cytometry but have not yet been characterized for this purpose. A case in point are the cryptomonad phycobiliproteins, a group of low- molecular weight proteins isolated from several genera and species of cryptomonad algae (7,8). A characteristic of cryptomonad phycobiliproteins that makes them particularly attractive as fluorescent labels is their relatively low molecular weights, between 30 and 60 kd (7,8). This molecular size is in contrast to R-PE and APC, which possess molecular weights between 214 and 290 kd and between 95 and 105 kd, respectively (1). High-molecular weight fluorochromes can be problematic in detection systems requiring conjugation to an antibody or other protein. This is because the largeness of the conjugate can both alter the conformation of the tagged protein and sterically hinder binding to its cognate. Although PE and APC are used frequently in extracellular immunolabeling, their use is sometimes precluded due to their size; low-molecular weight fluorescent haptens with lower quantum yields such as fluorescein must often be substituted in their place. High-molecular weight phycobiliproteins are also limited in their ability to detect intracellular antigens. The fluorochrome-antibody complex must cross a permeabilized cell membrane to reach its target. Phycobiliproteins are generally not used for the detection of intracellular proteins (such as cyclins). They are used for intracellular cytokine detection only because their high quantum yields outweigh their limited accessibility to the intracellular space (9). A group of phycobiliproteins that share many of the advantages of PE and APC yet possess a three- to fivefold lower molecular weight could find many applications in cellular analysis, particularly in flow and laser scanning cytometry.

In this study, we characterize a group of cryptomonad phycobiliproteins (commercially available and marketed as the CryptoFluor™ dye series) for their applicability to traditional flow cytometric analysis of extracellular and intracellular antigens. Several of these phycobiliproteins were comparable in performance to existing phycobiliprotein and synthetic fluorochromes, confirming their utility in flow cytometric analysis.

## MATERIALS AND METHODS

### Cells

Several cell types were used in these studies. EL4 mouse thymoma, HH human T lymphoma, and UMR-106 rat os-

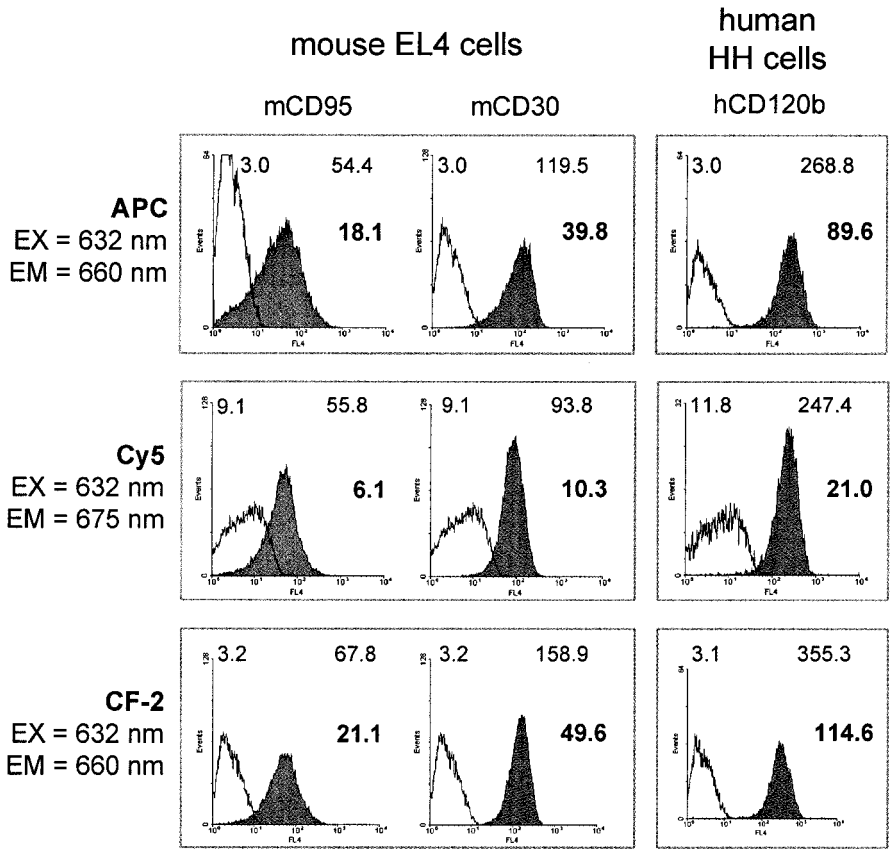


FIG. 2. Comparison of APC, Cy5, and CF-2 for extracellular immunophenotyping. Mouse EL4 cells were labeled with biotin-anti-CD95 or biotin-anti-CD30 **(left and middle)** or with human HH cells with biotin-anti-CD120b **(right)**. This was followed by labeling with either APC- (4 μg per sample), Cy5- (4 μg per sample), or CF-2–conjugated streptavidin (16 μg per sample). APC-, Cy5-, and CF-2–labeled cells were then excited with an HeNe 632-nm laser and fluorescence was detected with a 660 ± 20-nm filter (APCand CF-2, rows 1 and 3) or with a 675 ± 20-nm filter (Cy5, row 2). Mean fluorescence intensities (MFIs) are for isotype control (open peaks) and specific antibody labeling (filled peaks) is shown on each histogram. Ratios between specific and background MFIs are shown in boldface.

teosarcoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). They were serially passaged in RPMI 1640 with 10% fetal bovine serum (FBS). UMR-106 cells were adherent and required trypsin-EDTA removal prior to passage. Human peripheral blood mononuclear cells (PBMCs) were obtained from consenting donors by venipuncture and depleted of red blood cells (RBC) by centrifugation over Ficoll-Hypaque separation gradients. Once harvested, cells were immunolabeled and washed at 4°C with phosphate-buffered saline (PBS) containing 2% FBS and 0.1% sodium azide (henceforth termed label buffer). EL4 cells were labeled and washed in the above buffer without sodium azide, due to its toxicity to this cell line.

### Extracellular Immunolabeling

Harvested cells were subdivided into 12 × 75-mm culture tubes, washed with label buffer, decanted, and resuspended in one of the following biotinylated primary antibodies: anti-mouse CD95/Fas (clone Jo-2, BD Pharmingen, San Diego, CA), anti-mouse CD30 (clone mCD30.1, BD Pharmingen), anti-mouse CD90/Thy1.2 (clone 5a-8, Caltag Laboratories, Burlingame, CA), anti-mouse CD8 (clone 53-6.7, Becton-Dickinson Biosciences, San Jose, CA), anti-human CD120b/TNFR2 (clone 4D1B10/MR2-1, Caltag), anti-human CD3 (clone S4.1, Caltag), anti-human CD4 (clone S3.5, Caltag), or anti-human CD8 (clone 3B5, Caltag). Biotin-conjugated mouse, rat, and hamster isotype control antibodies were used for all control samples. All primary antibodies were used at a concentration of approximately 2 μg/ml with 100 μl containing $1 \times 10^6$ cells per well. Cells were incubated for 30 min at 4°C, washed twice with label buffer, and resuspended in one of the following streptavidin-conjugated fluorochromes: R-PE (Caltag), APC (Caltag), Cy3 (Jackson Immunoresearch, West Grove, PA), Cy5 (Jackson Immunoresearch), stabilized lissamine rhodamine (Rhodamine Red X, Jackson Immunoresearch), Alexa Fluor 568 dye (Molecular Probes, Eugene, OR), or one of three streptavidin-conjugated cryptomonad phycobiliproteins. These cryptomonad phycobiliproteins were CryptoFluor-2, CryptoFluor-4, and CryptoFluor-5.

**CryptoFluor-2.** CryptoFluor-2 is reported in the literature as Cr-$PC_{645}$ or phycocyanin 645. Cr-$PC_{645}$ is isolated from the alga *Chroomonas* sp. (7). CF-2 possesses a molecular weight of $4.02 \times 10^4$ daltons (estimated by gel permeation chromatography). This fluorochrome is composed of two subunits, an α subunit containing one 697B chromophore and a β subunit consisting of two PCB and one CV chromophore. This protein possesses several excitation peaks between 575 and 640 nm and has a peak emission at approximately 658 nm (0.1 mM sodium phosphate, 1 mM EDTA, pH 7.0; 10).

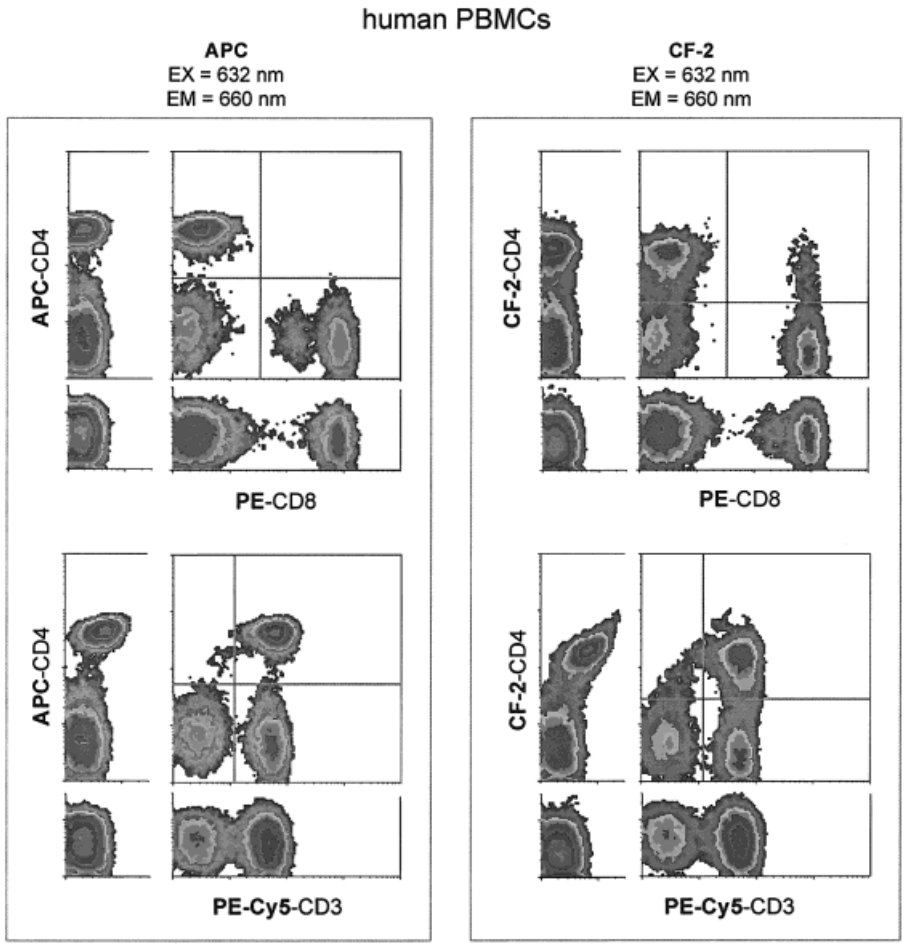


FIG. 3. CF-2 in multicolor labeling. Human PBMCs were immunolabeled with PE-anti-human CD8, PE-Cy5-anti-human CD3, and biotin-anti-CD4 followed by either APC- **(left)** or with CF-2–conjugated streptavidin **(right)** at the concentrations used in Figure 2. Cells were then analyzed by a dual-laser flow cytometer (argon 488 nm/HeNe 632 nm). Intra- and interlaser color compensation values were the same for APC- and CF-2–labeled samples.

**CryptoFluor-4.** CryptoFluor-4 is reported in the literature as Cr-$PE_{555}$ or phycoerythrin 555. Cr-$PE_{555}$ is isolated from *Hemiselmis* sp. (7). CF-4 has a molecular weight of $3.45 \times 10^4$ daltons as estimated by gel permeation chromatography. This fluorochrome is composed of two subunits present in an $\alpha_2\beta_2$ structure similar to that of CryptoFluor-3. This protein possesses an excitation maximum at 555 nm and has a peak emission at 580 nm (0.1 mM sodium phosphate, 1 mM EDTA, pH 7.0; 11).

**CryptoFluor-5.** CryptoFluor-5 is reported in the literature as Cr-$PE_{566}$ or phycoerythrin 566. Cr-$PE_{566}$ is isolated from *Chroomonas* sp. (7). CF-5 possesses a molecular weight of $3.08 \times 10^4$ daltons as estimated by gel permeation chromatography. This fluorochrome is reportedly composed of two subunits, an α subunit with one PEB chromophore and a β subunit consisting of one PEB chromophore. This protein possesses an excitation maximum at 565 nm and has a peak emission at approximately 600 nm (0.1 mM sodium phosphate, 1 mM EDTA, pH 7.0; 8).

Conjugation of CryptoFluor dyes to streptavidin and extraction of the resulting product were carried out by SATA/SMCC chemistry (Pierce Chemical, Rockford, IL) and Sephacryl S-200 (Amersham Pharmacia Biotech, Uppsala, Sweden) column purification as previously described (14); characterized conjugates were provided by Martek Biosciences (Columbia, MD). Final reagent concentration for immunolabeling varied depending on the fluorochrome. Cells were incubated in the secondary streptavidin-conjugated fluorochromes for 30 min at 4°C, followed by two washes in label buffer and analysis by flow cytometer within 1 h of labeling.

### Intracellular Labeling for Flow Cytometry

Following harvest, cells were washed once with PBS, decanted, resuspended in 70% EtOH, and stored at 4°C for 4–24 h. Cells were centrifuged and the pellets washed twice with label buffer, decanted, and resuspended in biotin-conjugated anti-PCNA (clone PC10) at 2 μg/ml in 100 μl volume containing $1 \times 10^6$ cells. Cells were incubated overnight at 4°C, washed twice, and resuspended in Cy5-, Cy3-, CF-2, or CF-4–conjugated streptavidin for 4 h at 4°C. Cells were washed twice with label buffer and analyzed by flow cytometry.

### Intracellular Labeling for Laser Scanning Cytometry

UMR-106 rat osteosarcoma cells were grown to partial confluency on glass tissue culture slides followed by aspi-

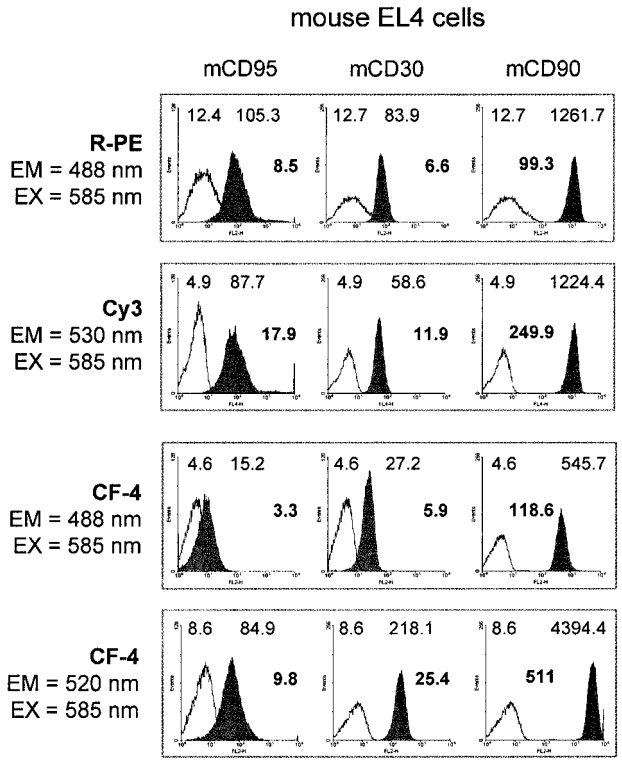


FIG. 4. Comparison of PE, Cy3, and CF-4 for extracellular immunophenotyping. Mouse EL4 cells were labeled with biotin-anti-CD95 **(left)**, biotin-anti-CD30 **(middle)**, or biotin-anti-CD90 **(right)**. This was followed by labeling with either R-PE- (4 μg per sample), Cy3- (4 μg per sample), or CF-4–conjugated streptavidin (16 μg per sample). R-PE–– and Cy3-labeled cells were excited with an argon 488-nm laser and fluorescence was detected through a 585 ± 26 filter (rows 1 and 2). CF-4 was excited with either an argon 488-nm (row 3) or a Kr 520-nm laser (row 4); fluorescence was detected through a 585 ±26 filter (rows 3 and 4). Mean fluorescence intensities (MFIs) for control (open peaks) and specific antibody labeling (filled peaks) are shown on each histogram. Ratios between specific and background MFIs are shown in boldface.

ration of tissue culture media and addition of 70% EtOH at 4°C. After incubation in EtOH for 4–24 h, cells were washed twice with label buffer and incubated overnight with biotin-conjugated anti-PCNA (5 μg/ml at 200 μl per well). Cells were then washed twice with label buffer and subsequently incubated with Cy5-streptavidin (Jackson Immunoresearch) or CF-2–conjugated streptavidin for 2 h at 4°C. Slides were washed twice and counterstained with propidium iodide (PI) 50 μg/ml containing RNase 100 U/ml. Coverslips were put on the slides and analyzed within 1 h.

### Data Acquisition and Analysis

Flow cytometry samples were analyzed on a FACSVantage SE cell sorter (BD Biosciences) equipped with a water-cooled argon-ion (6 W, I-90, Coherent, Santa Clara, CA), a krypton-ion (5 W, I-302C, Coherent), and an air-cooled HeNe laser (35 mW Model 127, Spectra-Physics, Mountain View, CA). This instrument received daily calibration with PE- and APC- conjugated Calibrite fluorescent particles (BD Biosciences) to ensure consistent day-to-day sensitivity. Argon-ion and krypton-ion laser power was set at 100 mW for all laser lines used in these experiments; the HeNe laser emitted at a fixed power of 35 mW. PE was excited using argon-ion 488-nm excitation and it was detected through a 575 ± 25-nm narrow band pass (NB) filter. Cy3, Alexa Fluor 532 dye, and CF-4 were excited using either argon-ion 488 or 514.5-nm or krypton-ion 520 or 530-nm laser lines and they were detected through a 585 ± 22 NB filter. APC, Cy5, and CF-2 were excited using either HeNe 632-nm or krypton-ion 568-nm laser lines and they were detected through a 630 ± 22 NB filter. Lissamine rhodamine, Alexa Fluor 568 dye, and CF-5 were excited with a krypton-ion 568-nm laser line and they were detected using a 610 ± 20 NB filter (Chroma Technology, Brattleboro, VT). Multicolor analysis using either APC or CF-2 in combination with R-PE and PE-Cy5 used dual-beam argon-ion 488-nm and HeNe 632-nm excitation and crossbeam compensation to separate PE-Cy5 and APC/CF-2 signals. All flow cytometry data were analyzed using CellQuest version 3.2 (BD Biosciences) and WinMDI version 2.8 (Dr. Joe Trotter, Scripps Institute) flow analysis software.

UMR-106 slides were analyzed with an LSC (Compucyte, Cambridge, MA) laser scanning cytometer equipped with argon-ion (488-nm) and HeNe (632-nm) lasers, with Cy5 or CF-2 and PI emission detected through 585 ± 25 and 650 LP filters, respectively. Laser scanning cytometry data were acquired and analyzed using WinCyte software (Compucyte).

## RESULTS

### Extracellular Immunolabeling With Cryptomonad Phycobiliproteins

Five cryptomonad phycobiliproteins described in the Materials and Methods were evaluated for their use in extracellular immunolabeling of viable cells with analysis by flow cytometry. Three (CF-2, CF-4, and CF-5) were particularly useful for flow cytometry. Their excitation and emission spectra are shown in Figure 1. CF-2 had excitation and emission spectra similar to those of APC or the cyanine dye, Cy5. Therefore, it was expected to be optimally excited by a red laser source (HeNe 632 nm or red diode 635 nm) and detected through intermediate red NB filters (630–690 nm). CF-4 had excitation and emission spectra similar to those of the cyanine dye, Cy3. We anticipated that it would be optimally excited by a green laser line (argon-ion 514.5 nm, krypton-ion 520 or 530 nm, or green diode 532 nm) with detection through an orange NB filter (570–610 nm). CF-5 has excitation/emission spectra similar to those of lissamine rhodamine, suggesting optimal excitation with a yellow laser source (such as krypton-ion 568 nm) and detection through a near-red filter (590–630 nm). These laser and filter combinations were tested for each fluor using several standard cell lines with known protein expression characteristics and with biotin-conjugated primary antibodies against these receptors. Although direct comparisons between fluorochrome "brightness" are affected by many factors other than the

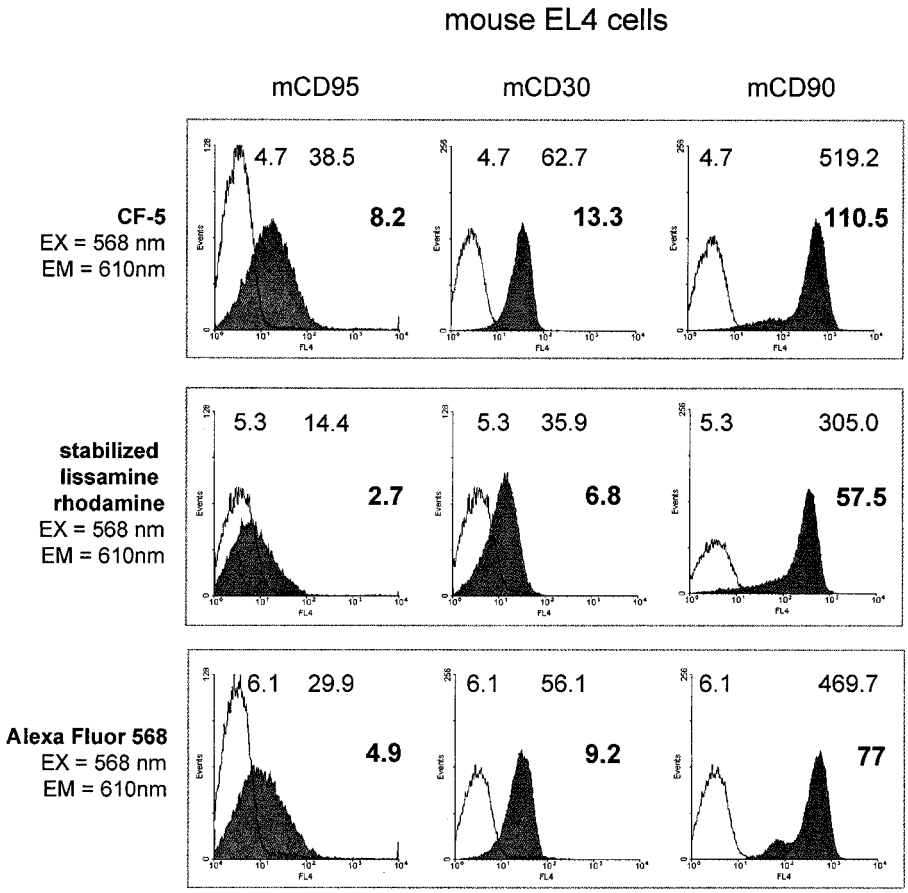


FIG. 5. Comparison of CF-5, lissamine rhodamine, and Alexa Fluor 568 dye for extracellular immunophenotyping. Mouse EL4 cells were labeled with biotin-anti-CD95 **(left)**, biotin-anti-CD30 **(middle)**, or biotin-anti-CD90 **(right)** followed by either CF-5- (16 μg per sample, row 1), lissamine rhodamine- (8 μg per sample, row 2), or Alexa Fluor 568-conjugated streptavidin (8 μg per sample). All fluorochromes were excited with a Kr 568-nm laser and fluorescence was detected through a 610 ± 30-nm filter (row 1-3). MFIs are for the control (open peaks) and specific antibody labeling (filled peaks) shown on each histogram.

absolute fluorescence yield of the molecule, these experiments give an estimate of the use of these markers relative to each other and to other commonly used fluors.

### CF-2

Extracellular immunophenotyping results for CF-2 are shown in Figure 2. Mouse EL4 thymoma and human HH lymphoma cells were labeled with biotin-conjugated anti-CD95 or CD30 and CD120b, respectively. This was followed by APC, Cy5, or CF-2-streptavidin secondary labeling. CF-2 excitation was tested with HeNe 632-nm and krypton-ion 568-nm laser sources and emission was tested with a 660/20-nm NB filter. CF-2 labeled the tested surface antigens with a fluorescence yield similar to that of APC and higher than that of Cy5 (with 675 ± 20 NB filter for optimal Cy5 detection; Fig., 2, rows 1–3). Optimal excitation/emission conditions for CF-2 were the same as those for APC, namely HeNe 632-nm excitation and detection through a 660 ± 20-nm NB filter. Excitation with shorter laser wavelengths such as a krypton-ion 568 gave lower but acceptable excitation, with a fluorescence yield that exceeded that of APC (data not shown). NB filters with longer wavelength characteristics showed diminished emission detection (data not shown). The cryptomonad phycobiliprotein, CF-1, has excitation/emission maxima that are at slightly shorter wavelengths than those for CF-2. The flow cytometric excitation/emission detection requirements for CF-1 were similar to those of CF-2, although the fluorescence yield was not as high as that of APC for extracellular labeling. This is likely the result of its blue-shifted excitation/emission maxima (data not shown).

The apparent equivalence between APC and CF-2 for extracellular labeling begged the question of whether CF-2 could replace APC in multicolor surface labeling experiments, a common application of APC. To this end, human PBMCs were immunolabeled with PE-conjugated anti-human CD8, PE-Cy5–conjugated anti-human CD3, and biotin-conjugated anti-human CD4, followed by either APC- or CF-2-streptavidin. The cells were then analyzed on a two-beam flow cytometer, using argon-ion 488-nm excitation for PE and PE-Cy5 and HeNe 632 nm for APC or CF-2 (Fig. 3, left and right columns, respectively, cells pregated on the small lymphocyte scatter subset). Particular spectral properties of APC make it applicable for multicolor labeling, specifically its low excitation at 488 nm, preventing signal "crosstalk" and minimizing fluorescence color compensation between it and PE-Cy5 (which emits in roughly the same range). In these experiments, CF-2 was essentially interchangeable with APC for multicolor labeling with PE and PE-Cy5. The level of interbeam

fluorescence compensation required using either APC/PE-Cy5 or CF-2/PE-Cy5 signal separation was slightly higher for CF-2 (<5%), suggesting that CF-2 can be readily substituted for APC in multicolor experiments.

### CF-4

Extracellular immunophenotyping results for CF-4 are shown in Figure 4. Mouse EL4 thymoma cells were labeled with biotin-conjugated anti-mouse CD95, CD30, or CD90 followed by R-PE (Fig. 4, row 1), Cy3 (Fig. 4, row 2), or CF-4-streptavidin (Fig. 4, rows 3 and 4) secondary labeling. CF-4 excitation was tested using argon-ion 488 or krypton-ion 520-nm laser lines. Fluorophore emission was evaluated with NB filters ranging from 585 nm. Interestingly, CF-4 showed detectable excitation at 488 nm (Fig. 4, row 3). Nevertheless, the longer wavelength laser lines were optimal, with the 520-nm krypton-ion line performing marginally better than 514.5 or 530 nm excitation (Fig. 4, row 4 and data not shown). Detection was optimal with a 585 ± 22 nm NB filter, with gradually longer wavelength filters showing a significant drop in detection sensitivity (data not shown). CF-4 under optimal conditions was as sensitive as R-PE for extracellular immunolabeling, although with different excitation requirements. CF-3 was similarly evaluated and possessed the same excitation/emission requirements as CF-4, although its fluorescence yield was lower than that for both R-PE and CF-4. It also possessed a significant level of nonspecific binding, which resulted in a "tailed" fluorescence pattern (data not shown).

### CF-5

Extracellular immunophenotyping results for CF-5 are shown in Figure 5. CF-5 (Fig. 5, row 1) was compared with stabilized lissamine rhodamine (Fig. 5, row 2) and Alexa Fluor 568 dye (Fig. 5, row 3), two low-molecular weight fluorochromes with similar excitation/emission requirements. CF-5 gave detection sensitivities equal to or exceeding those of lissamine rhodamine and Alexa Fluor 568 dye using krypton-ion 568- nm excitation and emission detection at 610 nm. Its extremely high fluorescence yield made it comparable in fluorescence to that of CF-2 and APC.

### Intracellular Labeling With CF-2 and CF-4

Intracellular immunolabeling is a potentially valuable application of the cryptomonad phycobiliproteins, given their relatively low molecular weights. Low-molecular weight synthetic organic fluorochromes (such as the cyanin dyes, Cy3 and Cy5) are usually optimal for intracellular labeling. To be useful in this application, the brightness of the CryptoFluor dyes should equal or exceed that of the Cy dyes. To determine if this was the case, mouse EL4 cells were permeabilized with 70% EtOH and immunolabeled with anti-PCNA antibody. This was followed by secondary labeling with Cy5 or CF-2–conjugated streptavidin (Fig. 6, top) or Cy3 and CF-4 (Fig. 6, bottom). All fluorophores gave good immunodetection of PCNA, although Cy5 gave better sensitivity than CF-2. CF-4, however, provided equal or better sensitivity than Cy3 for PCNA immunolabeling. CF-2 performed comparably to Cy5 in the laser scanning cytometry data (Fig. 7). UMR-106 rat osteosarcoma cells immunolabeled for PCNA and counterstained with PI showed equal brightness with either Cy5 or CF-2 (the differing distribution of $PCNA^{low}$ and $PCNA^{high}$ cells was due to unequal distribution of cells on the scanning surface, not to the sensitivity difference between the dyes).

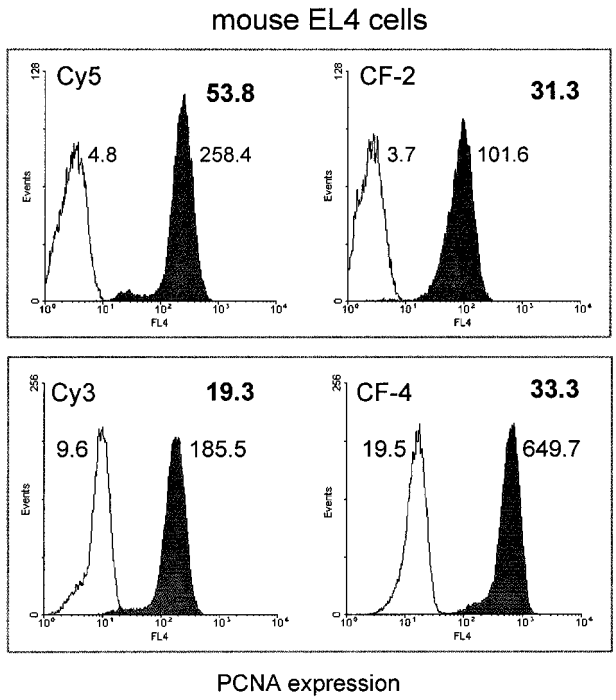


FIG. 6. Comparison of Cy5, Cy3, CF-2, and CF-4 for intracellular immunophenotyping. Mouse EL4 cells were fixed with 70% EtOH as described in the Materials and Methods and innunolabeled with biotin-conjugated anti-PCNA, followed by secondary labeling with Cy5-, Cy3-, CF-2–, or CF-4–conjugated streptavidin. Cy5- and CF-2–labeled cells were then excited with an HeNe 632-nm laser and fluorescence was detected through a 675 ± 20-nm (Cy5, **top left**) or a 660 ± 20-nm filter (CF-2, **top right**). Cy3- and CF-4–labeled cells were excited with a Kr 520-nm laser and fluorescence was detected through a 585/26-nm filter. MFIs are for control (open peaks) and specific antibody labeling (filled peaks).

## DISCUSSION

A series of cryptomonad algae-derived phycobiliproteins (commercially termed the CryptoFluor fluorochromes) were evaluated for their ability to detect both extracellular and intracellular antigens by flow cytometry. All of these fluorochromes were capable of excitation by wavelengths associated with standard flow cytometer laser sources, including argon- and krypton-ion green and yellow and HeNe red lines and were detected through standard NB filters. Three phycobiliproteins, CF-2, CF-4, and CF-5, were particularly applicable for flow cytometry and were studied in more detail. CF-2 gave a fluorescence yield similar to that of APC with HeNe 632-nm excitation. It was also compatible with 488 nm-excited fluoro-

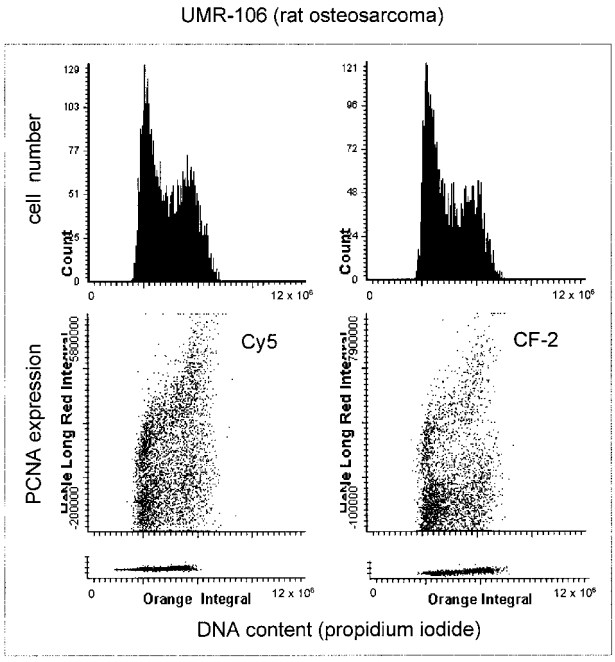


FIG. 7. Comparison of Cy5 and CF-2 for intracellular immunophenotyping by laser scanning cytometry. Rat osteosarcoma cells were grown on monolayers on glass tissue culture slides, fixed with 70% EtOH, and immunolabeled with biotin-conjugated anti-PCNA followed by secondary labeling with either Cy5- **(left)** or CF-2–conjugated streptavidin **(right)**. Cells were counterlabeled with PI 50 μg/ml followed by analysis with laser scanning cytometry using dual-laser excitation (argon 488 nm/HeNe 632 nm). DNA histograms (top), PCNA expression (middle), and controls (bottom) for Cy5 and CF-2 labelings. PCNA and DNA fluorescence measurements are shown in linear scale. PCNA measurements are normalized.

chromes including PE and PE-Cy5, making it applicable to multicolor analysis. The green-excited protein, CF-4, was exceptionally bright, yielding better fluorescence than either R-PE or Cy3. The yellow-excited protein, CF-5, was similarly brighter than dyes with similar excitation/emission characteristics. These fluorochromes are compatible with standard flow cytometric configurations because they spectrally emulate many fluorochromes currently used for flow cytometry (e.g., APC, Cy3, Cy5, lissamine rhodamine, and Alexa Fluor 568 dyes). The CryptoFluor fluorochromes share the advantages of other phycobiliproteins, namely, good photostability, long Stokes shifts, and good water solubility (7). Although these results limit themselves to streptavidin conjugates, other conjugates have also been prepared, including anti-mouse immunoglobulin, with good results (data not shown).

A potentially valuable application for CryptoFluor dyes is the detection of intracellular proteins, a technique that has been necessarily dominated by synthetic organic fluors. Although high-molecular weight phycobiliproteins such as R-PE and APC have been used for limited intracellular applications, they are hampered by their large molecular weights (>100,000 daltons). The comparatively low molecular weights of the CryptoFluor dyes make them useful candidates for intracellular labeling applications. In this study, CF-4 performed better than Cy3 for the immunodetection of cell cycle proteins.

## LITERATURE CITED

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