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Cy7PE and Cy7APC: Bright New Probes for Immunofluorescence

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We demonstrate the utility of indotricarbocyanine (Cy7) conjugates of the phycobiliproteins phycoerythrin (PE) and allophycocyanin (APC) in flow cytometry. This is the first demonstration of the use of an APC tandem dye for fluorescence measurements. These resonance energy transfer tandem dyes can be excited by the phycobiliprotein-specific excitation wavelengths and fluoresce at wavelengths above 780 nm. The tandem dyes, when conjugated to antibodies, are suitable for flow cytometry and other immunofluorescence applications. These conjugates are easily detectable above the very low autofluorescence in this part of the spectrum. Indeed, the Cy7-conjugated PE tandem (Cy7PE) has a "brightness" (fluorescence signal over cellular autofluorescence) comparable to that of fluorescein, and the Cy7APC tandem has a "brightness" comparable to that of APC. These tandems are also easily distinguished from other commonly used fluorophores, making them suitable for high-order multiparametric analysis. We show an example of six-color immunofluorescence analysis by flow cytometry, simultaneously measuring fluorescences from fluorescein, PE, Cy5PE, Texas red, APC, and Cy7APC. © 1996 Wiley-Liss, Inc.

Key terms: Phycoerythrin, allophycocyanin, cyanine, tandem dyes, resonance energy transfer, multiparameter measurements

There is always a need for more fluorophores (fluors) that can be used in immunofluorescence applications. New fluors should be bright (in comparison to cellular autofluorescence at the wavelength used to detect them), have minimal spectral overlap with other commonly used fluors, and be readily conjugated to monoclonal antibodies. These qualities are necessary for inclusion in immunofluorescence assays in which multiple probes are to be measured simultaneously. Successful fluors include small organic molecules, such as fluorescein or Texas red, and phycobiliproteins (PBP), such as phycoerythrin (PE) or allophycocyanin (APC).

Cellular autofluorescence is often a limiting factor in fluorescence detection. Therefore, considerable effort has been spent to develop red (or infrared) fluorescent dyes—those that emit at wavelengths longer than 600 nm. Waggoner and colleagues developed the fluorescent molecules indodicarbocyanine (Cy5) and indotricarbocyanine (Cy7) for this purpose (5,9). These fluors have many qualities that make them extremely useful: 1) They have a very high extinction coefficient, 2) they have a high quantum efficiency, and 3) they emit in the red (Cy5) and far red (Cy7). Consequently, they are very bright when used in immunofluorescence applications. In addition, Cy5 and Cy7 have relatively narrow excitation and emission bands that overlap only minimally with a variety of other fluors. This means that they can be used simultaneously with several other fluors with minimal correction for interchannel spectral overlap (i.e., fluorescence compensation).

Cy5, when conjugated to antibodies, requires excitation at wavelengths around 630 nm [as provided by, for instance, a helium-neon (HeNe) laser]. However, the conjugation of the Cy5 to the phycobiliprotein PE generates a resonance-energy transfer (RET) probe. When the emission spectrum of one fluor overlaps the excitation spectrum of another, and they are sufficiently close to each other (2–5 nm), then it is possible for the excitation energy of the first fluor to be transferred to the other through a nonradiative process termed RET (1). In

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this case, the first fluor is termed the donor, the second the acceptor. When Cy5 is covalently linked to PE, the tandem can be excited with 488 nm light (exciting the donor, PE), and fluorescence emitted by Cy5 (the acceptor) can be detected (3,8,10).

Cy5PE has become an important fluor in flow cytometric analyses. This tandem provides a very bright reagent that can be simultaneously used with fluorescein and PE with excitation from a single laser line (488 nm). These three fluors are commonly used in single-laser flow cytometers for three-color immunofluorescence.

Development of tandem reagents is generally more difficult than development of simple fluors, primarily because of the added variable of the ratio of acceptor to donor (A/D). In general, a high A/D yields the best transfer efficiency; however, at such ratios, the acceptor often self-quenches (resulting in lower fluorescence), and may cause the tandem to become "sticky" and thereby have undesired binding characteristics. Therefore, a compromise between optimal transfer efficiency and optimal acceptor fluorescence is necessary.

In this report, we show that Cy7 can be conjugated to either PE or to another PBP, APC. In the former case, the result is a tandem that can be excited at 488 nm (e.g., by an argon ion laser) but emits at 780 nm, a Stokes shift of 300 nm. The Cy7PE can be measured independently from FITC, PE, and Cy5PE, providing a fourth reagent that can be independently and simultaneously measured with a single excitation line. The Cy7APC tandem can be excited at 600–647 nm and also emits at 780 nm. This bright dye provides a third color for dye laser (600 nm) excitation and a second color for HeNe (633 nm) excitation. These tandems are bright (with respect to cellular autofluorescence) and do not have significant nonspecific binding characteristics.

MATERIALS AND METHODS.

Materials

We obtained succinimidyl 4-(N-maleimidomethyl)cyclohexane 1-carboxylate (SMCC) from Pierce (Rockford, IL), N,N′-biscarboxypentyl-5,5′-disulfonatoindotricarbocyanine (bis-Cy7; molecular weight 1001) from Amersham Life Sciences (Pittsburgh PA), N-ethylmaleimide (NEM) from Calbiochem (La Jolla, CA), and anhydrous dimethyl sulfoxide (DMSO) from Aldrich (Milwaukee, WI). The DMSO is kept desiccated at all times. We isolated PE and APC as previously described (2); the proteins are stored as saturated ammonium sulfate precipitates. We obtained all other chemicals from Sigma (St. Louis, MO).

Protein Conjugations: General Hints

Conjugations are optimal when the antibody concentration and the PBP concentration are at least 2 mg/ml. Do not add sodium azide to any of the buffers listed below (except, if desired, the final storage buffer); however, the use of pentachlorophenol (pHix®) as a preservative does not interfere with the conjugations. Never freeze solutions containing purified or conjugated PBP. It is often helpful to remove aggregates by centrifugation (5,000*g* for 10 min) or gel filtration prior to staining cells. This is especially true for PE conjugates.

Cy7PBP Conjugations

PE or APC was dialyzed into carbonate buffer (500 mM sodium carbonate, pH 9.0), with a resulting concentration of at least 5 mg/ml. Absorbance spectra of the PBP were used to determine concentration as well as purity, i.e., contaminants such as phycocyanin (PC): For APC, 1 mg/ml has an absorbance at 655 nm of 5.9; a ratio of absorbances at 655 to 620 of greater than 1.4 indicates adequate removal of PC, and a ratio of absorbances at 655 to 280 of greater than 4 indicates adequate removal of other proteins (2). For PE, 1 mg/ml has an absorbance at 565 of 8.2; a ratio of absorbances at 565 to 620 of greater than 50 indicates adequate removal of PC, and a ratio of absorbances at 565 to 280 of greater than 5 indicates adequate removal of other proteins. Consistent conjugations with Cy7 require that the PBP and the Cy7 be at the same concentration every time.

bis-Cy7 was dissolved in anhydrous DMSO at an effective concentration of 10 mg/ml (10 mM reactive dye) immediately prior to use. The appropriate amount of bis-Cy7 (see Table 1) was added to the PBP; the reaction tube was rotated at room temperature for 60 min. The Cy7-conjugated PBP (Cy7PBP) was purified by passing the reaction mixture over a PD-10 column preequilibrated with phosphate buffer (50 mM sodium phosphate, 1 mM EDTA, pH 7.0).

The concentration of the Cy7PBP tandem was once again determined spectrophotometrically (using the PBP-specific absorption bands). Also, the relative absorbance of the Cy7 (measured at 755 nm to the PBP) was calculated to ensure an appropriate degree of conjugation (see Results), with the assumption that the extinction coefficient of the dyes was unaltered by the conjugation procedure. A fresh solution of 10 mg/ml SMCC in anhydrous DMSO was prepared; 11 μl per mg PE or 6 μl per mg APC was added to the Cy7PBP. The reaction tube was rotated for 60 min at room temperature. The derivatized Cy7PBP was purified by passing the reaction mixture over a PD-10 column preequilibrated with morpholinoethylsulfonic acid (MES) buffer (50 mM MES, 2 mM EDTA, pH 6.0). The derivatized Cy7PBP is stable for extended periods (at least 8 weeks) if kept refrigerated (not frozen); we recommend the addition of pHix to preserve the material.

Antibody-Cy7PBP Conjugations

The IgG to be conjugated was reduced with dithiothreitol (DTT). A fresh solution of 1 M DTT in purified water was prepared; 20 μl of this solution was added per milliliter of IgG to make the concentration 20 mM DTT. This solution was gently mixed and then left standing at room temperature for 30 min. The reduced IgG was purified by passing over a PD-10 column preequilibrated with MES buffer. The purified IgG was immediately conjugated.

In general, optimal conjugates are obtained at IgG con-

centrations in excess of 2 mg/ml and PBP concentrations in excess of 2 mg/ml. We used 3.2 mg of Cy7PE or 1.5 mg of Cy7APC per mg IgG. The reactants (both in MES buffer) were mixed and then rotated at room temperature for 60 min. A fresh solution of 10 mg/ml NEM in anhydrous DMSO was prepared; 3.4 μl of this solution for every 1 mg IgG was added to the reaction mixture, and rotation was continued for another 20 min at room temperature. Finally, the Cy7PBP-IgG conjugate was purified by passing over a PD-10 column preequilibrated with an antibody storage buffer (for instance, 10 mM Tris, 150 mM sodium chloride, pH 8.0, with pHix®).

Cell Staining and Analysis

Peripheral blood mononuclear cells (PBMC) were obtained by standard methods; at least 10^6 cells were used for each stain. Antibodies (conjugated and unconjugated) were obtained from PharMingen (La Jolla, CA), except for fluorescein isothiocyanate (FITC) Leu7 (CD57) from Becton Dickinson (San Jose, CA). Cells were stained on ice for 15 min with fluorescently conjugated antibodies and then washed three times with staining medium (biotin, flavin-deficient RPMI medium supplemented with 4% fetal calf serum and 0.02% sodium azide). Routine fluorescence-activated cell sorting (FACS) analyses were performed on a modified FACStarPlus. Cy7 fluorescence was collected with a gallium-arsenide photomultiplier tube (R636; Hamamatsu), using a 50-nm-wide bandpass filter centered at 785 nm. Data were collected by FACS-Desk (4). Six-color analysis, requiring (interlaser) software compensation, was performed using software developed in our laboratory (Adam Treister, Stanford University).

RESULTS

We conjugated Cy7 to either PE or APC in an attempt to make novel RET dyes (tandems) for use in flow cytometry. The general method was to use a primary amino-reactive Cy7-reactive dye (i.e., the succinimidyl ester) to react directly with the PBP. The Cy7PBP tandem was then conjugated with the heterobifunctional linking reagent SMCC (also reacting with primary amines). The SMCC moiety also has a sulfhydryl-reactive maleimidyl group, allowing for site-specific conjugation to monoclonal IgG after the reduction of the immunoglobulin hinge disulfide. This manner of IgG conjugation results in a predictable ratio of PBP to IgG as well as rarely (if ever) interfering with the antigen-combining region.

The primary variable in making these tandems is the ratio of Cy7 (acceptor) to PBP (donor). The highest transfer efficiency will be obtained at the highest ratios. However, as the number of Cy7 molecules per PBP increases, the potential for self-quenching becomes greater, resulting in less net Cy7 fluorescence. In addition, solubility of the tandem may become a problem.

Therefore, we titrated the reactive Cy7 dye in the conjugation reaction to obtain a range of Cy7 to PBP ratios (Table 1). As shown is in Figure 1, the absorbance spectra of the tandems reflect the presence of both the Cy7 and the PBP moieties. The ratio of the PBP-specific absorbance band (565 nm for PE, 655 nm for APC) to the Cy7-specific absorbance band (755 nm) is useful for calculating the relative amounts of these two fluors present in a mixture.

Table 1
Cy7PBP Conjugations[a]

[PBP][b] (mg/ml)	[bis-Cy7][b] (μM)	Input Cy7:PBP (molar ratio)	Conjugate Cy7:PBP[c] (absorbance ratio)
Cy7APC conjugations			
6.3	260	4.6	0.59
6.3	390	6.9	0.84
6.3	530	9.2	1.20
5.4	340	7	1.03
5.4	490	10	1.37
5.4	640	13	1.77
5.4	840	17	2.38
5.4	1,080	22	3.80
8.3	760	10	1.86
Cy7PE conjugations			
10	620	15	0.53
10	500	12	1.10
10	380	9	0.32
10	620	15	1.21
9.4	1,180	30	3.70
9.4	590	15	0.93
9.4	390	10	0.58
10	500	12	1.25
10	620	15	2.22
10	750	18	2.63
10	880	21	3.22
10	330	8	0.62
10	420	10	0.95
10	500	12	1.47
10	580	14	2.00

[a]In general, there is a very good correlation between the input ratio of Cy7 to PBP and the resulting absorbance ratio, suggesting that the available reactive sites on the PBP were not being saturated with Cy7 at these molar ratios.
[b]Concentrations of APC, PE, or Cy7 during the first conjugation reaction. Generally, the total volume of the reaction was 0.5–1.0 ml (3–5 mg PBP total).
[c]Ratio of absorbances at 754 nm (Cy7) to 654 nm (APC) or 565 nm (PE).

The absorbance spectra of Cy7PE conjugates do not show appreciable differences from what would be obtained from an unreacted mixture of the molecules (Fig. 1). The PE-specific absorbance bands are identical for both the unconjugated PE and the tandem; the Cy7 absorbance band is slightly red shifted (5–10 nm), which is commonly observed for cyanines conjugated to proteins (L. Ernst, personal communication). There is also a slight increase in the relative absorbance at 700 nm, which is indicative of Cy7-dimer formation.

The spectra of the Cy7APC conjugates are also similar to those of the unconjugated molecules (Fig. 1). However, for the APC-specific absorbance, there is a decrease at 656 nm relative to 630 nm. This change is specific for APC conjugated to Cy7; it was not observed for APC conjugated to antibody or for APC reacted with SMCC prior to conjugation with Cy7 (data not shown). Regard-

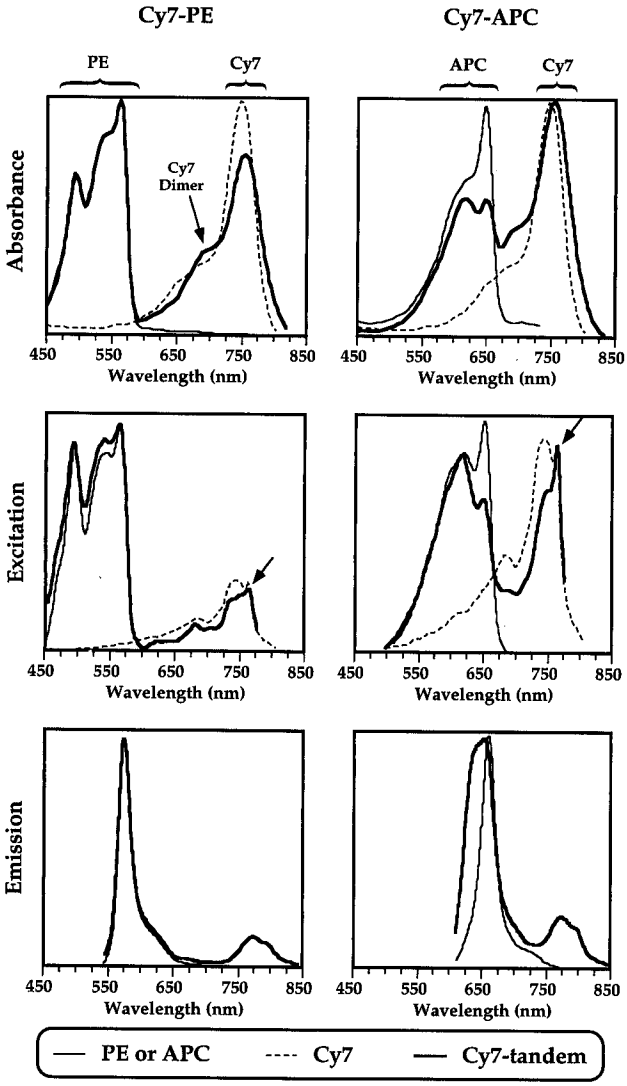

FIG. 1. Absorbance and fluorescence spectra of Cy7, PE, APC, and Cy7PBP tandems. The left panels focus on the Cy7PE tandem conjugates, the right panels on the Cy7APC tandem conjugates. Except for Cy7, all spectra were taken from dyes conjugated to immunoglobulin. The absorbance spectra (top panels) show a shoulder at 700 nm that is characteristic of Cy7-dimer formation; because Cy7 dimers are much less fluorescent, conjugation conditions should be selected to minimize this peak. The excitation spectra of the Cy7 tandems (middle panels) both show a significant increase of a very narrow, red-shifted excitation band of the Cy7 moiety for both PBP (~70 nm; arrows); this band may indicate the presence of highly fluorescent aggregates (see text). Emission spectra (bottom panels) demonstrate that RET is observed between the PBP and Cy7 when covalently linked. This proves that the overlap in the emission spectrum of the PBP and the excitation spectrum of the Cy7 is sufficient for this process to occur. Notes: Excitation and emission spectra are uncorrected for detector sensitivity. The excitation spectrum of PE was measured at 590 nm, of APC at 750 nm, and of Cy7 and Cy7PBP tandems at 850 nm. The emission spectra of PE and Cy7PE were measured at 488 nm, those of APC and Cy7APC at 590 nm. Spectra are independently scaled for presentation purposes; the ordinate is a linear scale.

ing the Cy7PE tandem, the Cy7 moiety shows a slight red shift in absorbance after conjugation.

The fluorescence spectra of the tandems (Fig. 1) demonstrate the RET that can be achieved when the Cy7 is within 2–5 nm of the PBP (e.g., covalently coupled). The spectra of the tandems (shown for immunoglobulin conjugates) are essentially unchanged compared to those of tandems not conjugated to immunoglobulins (data not shown). The PE-specific emission and excitation bands are essentially identical for the tandem and the unconjugated PE. For the APC tandem compared to unconjugated APC, there is a reduction in the 656 nm excitation band and a slight blue shift of the APC-specific emission spectrum. Most importantly, both tandems show Cy7 emission (i.e., >780 nm) when the PBP is excited. For Cy7PE, this represents a Stokes shift of 300 nm.

The excitation spectrum of the Cy7 moiety when conjugated to the PBPs shows a red shift. In particular, the spectrum is biased towards a very narrow excitation peak centered at approximately 760 nm; the peak at 740 nm is relatively diminished. Together with the mild bathochromic shift in the absorbance spectrum, this suggests the presence of "J" aggregates (L. Ernst, personal communication). These aggregates tend to display a slight red shift in excitation and a significant increase in fluorescence in comparison to monomer fluors.

In the unmodified APC molecule, there are two fluors with primary excitations at 630 nm and 650 nm. The RET from the 630 fluor to the 650 fluor is very efficient. Two changes in the APC spectra of the Cy7APC tandem suggest that the Cy7 moiety is interacting with the 650-excited fluor. First, both the absorbance and excitation of this fluor are diminished approximately 30–50% in comparison to the 630 band. Second, the APC-specific emission in the tandem is significantly blue shifted, suggesting that the 630-excited fluor is directly emitting (as opposed to transferring to the other APC fluor) much more frequently than in the unconjugated molecule. This may also explain the incomplete quenching of the APC fluorescence by Cy7 (see below).

We conjugated the different tandem PBP dyes (with different Cy7:PBP ratios) to monoclonal antibodies directed against human and mouse peripheral blood cell antigens (e.g., antihuman CD5). We evaluated the different Cy7 conjugates based on flow cytometric analysis of cells stained with the antibody conjugates.

Figure 2 shows some typical uncompensated contour plots of cells stained with these tandem conjugates of human anti-CD5. As these plots show, different ratios of Cy7 to PBP result in significantly different brightnesses in both the Cy7 channel (acceptor fluorescence) and the PBP channel (donor fluorescence). In general, as the ratio of Cy7 to PBP increases, the donor fluorescence decreases as a result of increased efficiency of energy transfer. Unfortunately, as the ratio of Cy7:PBP increases above a certain point, the total Cy7 fluorescence begins to decrease, suggesting that significant self-quenching occurs. The relationship of brightness and transfer efficiency to the ratio of Cy7 to PBP is shown in Figure 3.

Similar to the case with Cy5PE tandems, enough Cy7 can be conjugated to PE that the PE fluorescence is essentially completely quenched, and only Cy7 fluores-

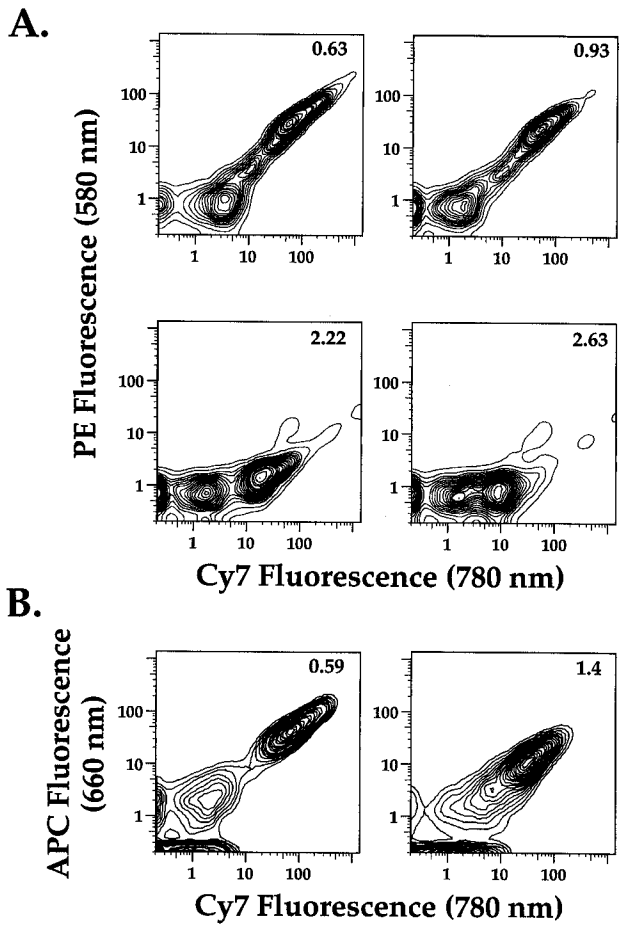

FIG. 2. Sample contour plots for both conjugates. PBMC were stained with CD5 conjugated to different preparations of Cy7PE (**A**) or Cy7APC (**B**). Shown are 5% probability contour plots of the uncompensated PBP signal (ordinate) and Cy7 signal (abscissa). Different ratios of Cy7 to PBP were used in these preparations; the ratio of Cy7 absorbance to PBP absorbance for the conjugate is given in the upper right corner of each panel. At lower ratios of Cy7 to PBP, higher Cy7 fluorescence can be seen, but greater compensation is required as well (i.e., higher PBP fluorescence is seen). For PE, enough Cy7 can be conjugated to quench essentially all PE fluorescence, with retention of some Cy7 fluorescence. Cy7 fluorescence was collected with a 785/50 filter (50 nm bandpass centered at 785 nm), PE with a 575/26 filter, and APC with a 660/22 filter.

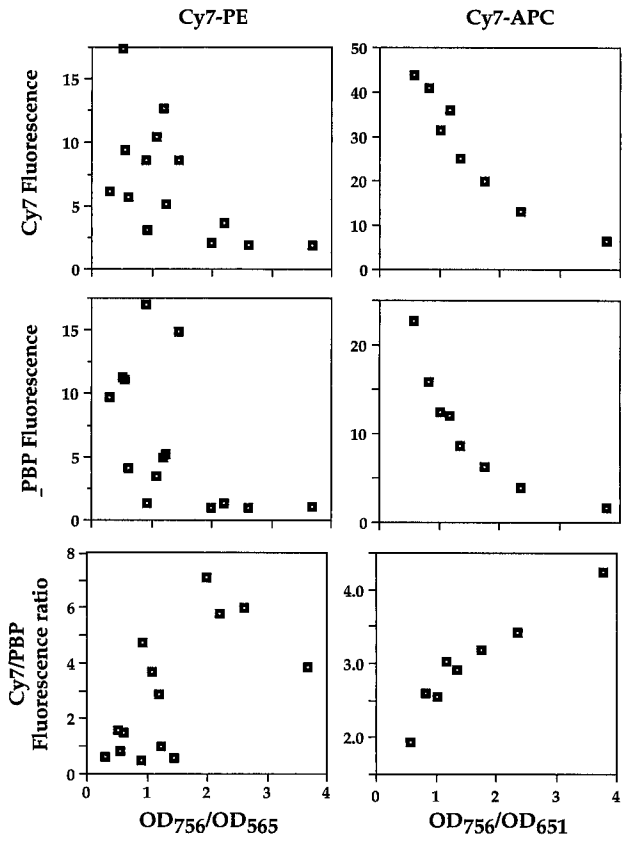

FIG. 3. Plot of brightness vs. conjugation ratio. Various preparations of Cy7PE (left) and Cy7APC (right) conjugated to CD5 were tested by flow cytometry. The absorbance ratio measured at the Cy7 band to the PBP band is proportional to the Cy7 to PBP molar ratio. **Top:** The median fluorescence in the Cy7 emission channel for the positively stained cells is shown as a function of the ratio of absorbance of the Cy7 to the PBP (i.e., the conjugation ratio). Note that, as the Cy7 to PBP ratio drops to zero, the Cy7 fluorescence must also drop to zero: thus, the curve must begin at the origin. At conjugation ratios above a certain point, the Cy7 density (on the PBP) is high enough that self-quenching occurs; hence, the Cy7 fluorescence decreases. **Middle:** The median fluorescence in the PBP emission channel for the positively stained cells. **Bottom:** The ratio of the Cy7 fluorescence to the PBP fluorescence. The inverse of this ratio is approximately equal to the compensation (i.e., the magnitude of the cross-channel spillover correction) required to normalize these channels.

cence remains. (At this point, the Cy7 fluorescence is significantly dimmer than at lower conjugation ratios). However, for APC, complete quenching was not observed. This may be a result of the fact that the 630 fluor of APC is no longer efficiently transferring energy to the 650 fluor and does not efficiently transfer to the Cy7 moeity. In the case of the PE tandem, the Cy7 conjugation has not interfered with the intramolecular RET between fluors (there are no significant changes in the spectra of the unmodified and Cy7-conjugated PE molecules); thus, complete quenching is attained at high Cy7 conjugation ratios. In any case, with our flow cytometric configuration, the Cy7APC conjugate can be easily resolved from APC conjugates with instrument compensation values less than 50%.

To demonstrate the utility of this dye in multiparametric analysis, we simultaneously stained human PBMC with six different fluorescent conjugates. Each of the six fluors can be independently (i.e., orthogonally) measured and quantitated, allowing (in this case) many different subsets of T cells to be simultaneously identified and enumerated.

Figure 4 shows an example of such a six-color experiment, one designed to explore the phenotype of the $CD57^+$ T cells in peripheral blood. We stained cells with CD3, CD4, and CD8 to identify uniquely and resolve the T cell lineages. In addition, we used CD45RA and CD62L,

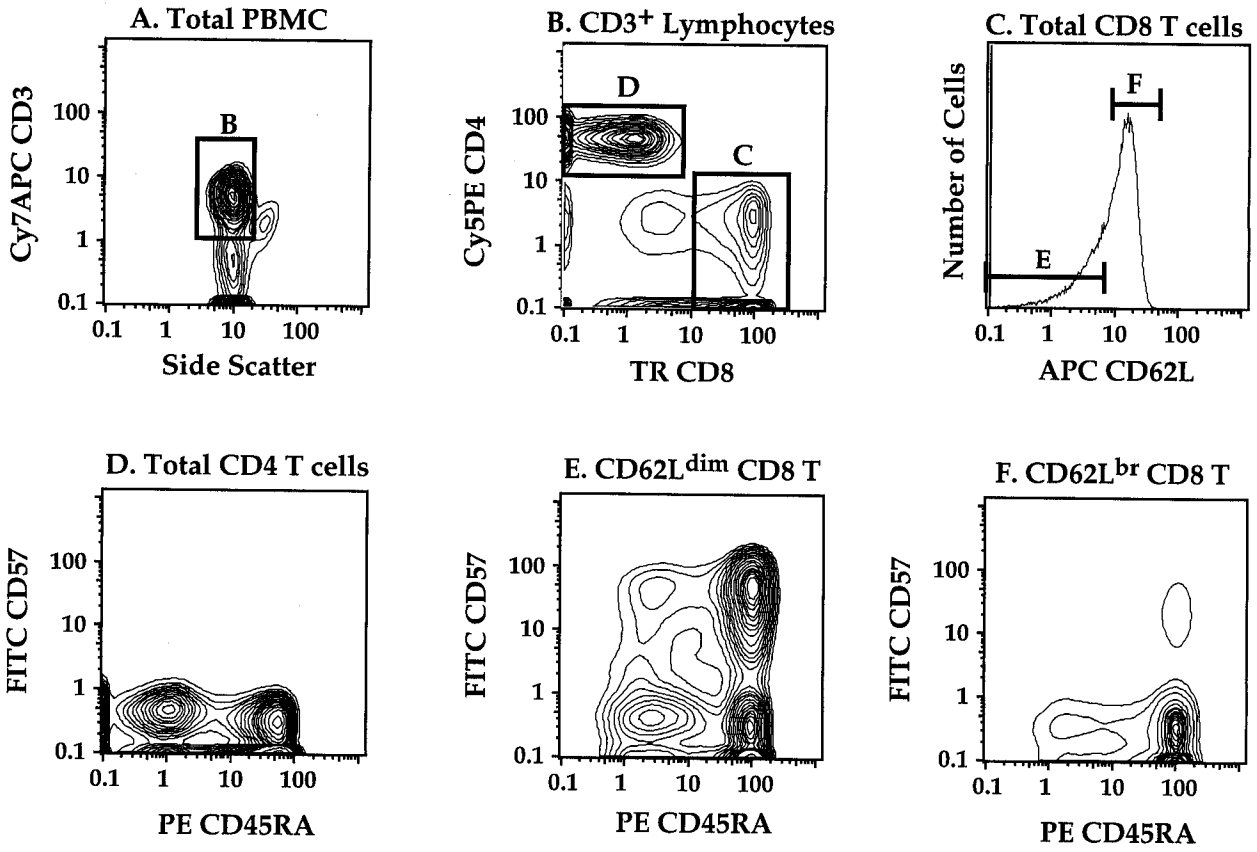

FIG. 4. Six-color staining of T cells. PBMC from a healthy adult donor were stained with FITC CD57, PE CD45RA, Cy5PE CD4, Texas red (TR) CD8, APC CD62L, and Cy7APC CD3. The letter designating each plot (for B–F) corresponds to the letter of a gate used to define those cells; for example, the cells in panel B are defined by gate B in panel A. The vast majority of $CD57^+$ T cells express CD8 but not CD4 and express high levels of CD45RA, but low levels of CD62L, a phenotype consistent with a specific subset of memory CD8 T cells.

which can identify stages of differentiation of mature T cells [i.e., naive T cells are $CD45RA^+$ and CD62L-bright; memory cells are either $CD45RA^-$ or CD62L-dim (6,7)]. The final antibody in the six-reagent combination was CD57. Progressive gating shows that the CD57 expression is restricted to CD8 T cells and, in particular, to memory CD8 T cells. In fact, the $CD57^+$ cells belong almost exclusively to the class of memory CD8 T cells that express CD45RA (but are dim for CD62L). This is another example of the failure of CD45RA by itself to identify uniquely naive or memory T cells.

DISCUSSION

Conjugation of PE or APC with Cy7 results in new fluorescent probes that can be excited with PBP-specific laser lines (i.e., 488–530 nm for PE, 600–650 nm for APC) and emit at wavelengths longer than 780 nm. Relative to autofluorescence, immunoglobulin conjugates of these tandems are as bright or brighter than fluorescein conjugates. The Cy7 tandems also are easily distinguished from other commonly used fluors.

Cy7 conjugation of PE does not significantly alter the PE molecule itself, as evidenced by the absence of change in the PE spectral characteristics. However, Cy7 conjugation of APC apparently interferes with the fluorescence properties of one of the internal APC fluors. This may be why we did not observe complete quenching of APC fluorescence, even at relatively high Cy7 conjugation ratios, and in spite of the fact that the spectral overlap of the Cy7 excitation is much greater with the emission of APC than of PE. It is possible that other red-excited phycobiliproteins (such as phycocyanin) may be conjugated with Cy7 in a manner that completely quenches their fluorescence, while they still retain significant Cy7 fluorescence.

Variation of the ratio of Cy7 to PBP results in significantly different dyes. In general, the brighter (Cy7 fluorescence) tandems also require more compensation with either APC or PE; lower compensation can be achieved at the expense of brightness. Therefore, the choice of an optimal ratio of Cy7 to PBP will depend on the needs of the application: When sensitivity is not an issue, a conjugate with a small compensation (but not very bright) might be chosen; for low-density antigens, a conjugate that is very bright (but requires greater compensation) might be chosen.

For our laboratory, we chose a Cy7 to PBP ratio that results in a reagent that is intermediate in brightness and

intermediate in the amount of compensation required. Because coupling of the tandem to monoclonal antibodies does not change the ratio of Cy7 to PBP fluorescence, we prepared a large quantity (30 mg) of this conjugate to allow for conjugation of many monoclonal antibodies; therefore, a constant compensation value can be used for all these conjugates. We have successfully conjugated the tandems to over a dozen monoclonal antibodies.

The Cy7PE dye has a significant advantage over Cy5PE when used on two-laser cytometers. Insofar as the Cy7PE tandem has little or no excitation at either 600 nm (dye lasers) or 633 nm (HeNe laser), it can be used in conjunction with APC without requiring substantial interlaser compensation. The Stokes shift (300 nm) is so large that this first-laser-excited tandem essentially "bypasses" the second laser.

Another advantage over Cy5PE is that the Cy7 tandems do not have the unusual "nonspecific" binding that has been observed with Cy5PE (when staining human PBMC). It has been suggested that human monocytes might have a "Cy5" receptor, insofar as they have very high background binding of Cy5PE that is attributable to the Cy5 moeity. Cy7PBP conjugates of antibodies that do not react with human monocytes do not show any increased fluorescence on monocytes (data not shown). This is a significant advantage of the Cy7 tandems over Cy5PE when studying PBMC or other cell types that bind Cy5 nonspecifically.

A significant application of the Cy7APC conjugate is on newer two-laser cytometers, such as the FACSCalibur®. This instrument collects three distinct fluorescent emissions from the 488 nm laser and one from a 633 nm laser. Typically, four-color experiments on this machine use FITC, PE, PerCP, and APC. Cy5PE as a third color on the first laser is not very useful: It is efficiently excited by the second laser and emits with a spectrum very similar to that of APC; i.e., it requires significant cross-laser compensation. However, using Cy7APC instead of APC (and using a 780 nm bandpass filter) removes this drawback: The amount of Cy5PE fluorescence in the Cy7 channel is quite small, and little or no compensation is required. This allows the use of three very bright fluorescent dyes (PE, Cy5PE, and Cy7APC) simultaneously with FITC. For this application, a ratio of Cy7 to APC would be chosen to give the maximal Cy7 fluorescence, because the amount of APC emission by the tandem would be irrelevant.

Of course, another significant application of the Cy7APC is in higher order multicolor experiments. The Cy7APC provides a third color emission from a 600 nm laser line (e.g., that from a dye laser). Thus, together with Texas red and APC, the Cy7APC tandem results in a three-color single-laser system that is very similar to the combination FITC, PE, and CY5PE. An example of this six-color staining is shown in Figure 4.

We have already extended this system in two ways: 1) to collect the Cy7PE fluorescence and 2) with a 400-nm-excitable dye together with a third laser. This system allows us to collect eight colors (and two scattered light parameters) for immunofluorescence experiments (Roederer et al., in preparation). Finally, because the Cy7PE can be excited independently of the second laser dyes, we can simultaneously measure seven fluors from just the two visible lasers (488 nm and 600 nm).

The Cy7PBP tandems are bright fluors that are easily coupled to monoclonal antibody reagents. These reagents are well suited to a variety of fluorescence applications, including flow cytometry and microscopy.

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LITERATURE CITED

1. Glazer AN, Stryer L: Fluorescent tandem phycobiliprotein conjugates. Emission wavelength shifting by energy transfer. Biophys J 43:383–386, 1983.
2. Hardy RR: Purification and coupling of fluorescent proteins for use in flow cytometry. In: Handbook of Experimental Immunology, 4th ed, Weir DM, Herzenberg LA, Blackwell C, Herzenberg LA (eds). Blackwell Scientific Publications, Boston, 1986, pp 31.1–31.12.
3. Lansdorp PM, Smith C, Safford M, Terstappen LW, Thomas TE: Single laser three color immunofluorescence staining procedures based on energy transfer between phycoerythrin and cyanine 5. Cytometry 12:723–730, 1991.
4. Moore WA, Kautz RA: Data analysis in flow cytometry. In: Handbook of Experimental Immunology, 4th ed, Weir DM, Herzenberg LA, Blackwell CM, Herzenberg LA (eds). Blackwell Scientific Publications, Boston, 1986, pp 30.1–30.11.
5. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS: Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. Bioconjug Chem 4:105–111, 1993.
6. Okumura M, Fujii Y, Takeuchi Y, Inada K, Nakahara K, Matsuda H: Age-related accumulation of LFA-1^{high} cells in a CD8 + $CD45RA^{high}$ T cell population. Eur J Immunol 23:1057–1063, 1993.
7. Picker LJ, Treer JR, Ferguson DB, Collins PA, Buck D, Terstappen LW: Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selection on T cells during the virgin to memory cell transition. J Immunol 150: 1105–1121, 1993.
8. Shih CC, Bolton G, Sehy D, Lay G, Campbell D, Huang CM: A novel dye that facilitates three-color analysis of PBMC by flow cytometry. Ann NY Acad Sci 677:389–395, 1993.
9. Southwick PL, Ernst LA, Tauriello EW, Parker SR, Mujumdar RB, Mujumdar SR, Clever HA, Waggoner AS: Cyanine dye labeling reagents—Carboxymethylindocyanine succinimidyl esters. Cytometry 11:418–430, 1990.
10. Waggoner AS, Ernst LA, Chen CH, Rechtenwald DJ: PE-CY5. A new fluorescent antibody label for three-color flow cytometry with a single laser. Ann NY Acad Sci 677:185–193, 1993.