

Phycoerythrin-Allophycocyanin: A Resonance Energy Transfer Fluorochrome for Immunofluorescence

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Background: As immunofluorescence experiments become more complex, the demand for new dyes with different properties increases. Fluorescent dyes with large Stoke's shifts that are very bright and have low background binding to cells are especially desirable. We report on the properties of the resonance energy tandems of phycoerythrin and allophycocyanin (PE-APC). PE-APC is the original fluorescence resonance energy tandem dye described in the literature, but it has not been utilized because of the difficulty of synthesizing and preparing a consistent product.

Methods: PE-APC complexes comprising different ratios of the two phycobiliproteins conjugated to streptavidin were synthesized using standard protein-protein conjugation chemistry. The PE-APC streptavidins were evaluated for flow cytometric analysis. They were compared directly to Cy5PE conjugates because Cy5PE is the fluorophore that is spectrally most like the PE-APC.

Results: PE-APC complexes showed the expected fluorescence spectral properties of a tandem: excitation was excellent at 488 nm (and best at the PE excitation maximum) and emission was greatest at the APC emission maximum at about 660 nm. The efficiency of transfer of energy from PE to APC was about 90%.

Conclusion: PE-APC can be considered an excellent substitute for Cy5PE. Compared with Cy5PE, PE-APC has similar brightness (in staining experiments), slightly greater compensation requirements with PE but much lower compensation with Cy5.5PE or Cy5.5PerCP, and lower nonspecific background binding. PE-APC is a useful alternative to Cy5PE, especially in applications in which the use of Cy5 is impractical. Cytometry 44:24–29, 2001. Published 2001 Wiley-Liss, Inc.[†]

Key terms: phycobiliproteins; immunophenotyping; fluorescent dyes; antibody conjugation

Immunofluorescence analysis of cells becomes significantly more powerful with the simultaneous use of multiple distinct fluorochrome-conjugated antibodies. The utility of these conjugates depends on several factors: (1) relative brightness (i.e., how much signal is detected on stained versus unstained cells), (2) background binding (i.e., the degree to which the conjugated antibody binds nonspecifically to cells compared with unconjugated antibody), and (3) spectral overlap (i.e., how well resolved the fluorescence excitation and emission spectra are from those of other fluorophores).

Resonance energy transfer (RET) tandem dyes, which consist of small inorganic molecules (such as indocarbocyanine [Cy5] or indotricarbocyanine [Cy7]) conjugated to phycobiliproteins (such as phycoerythrin [PE] or allophycocyanin [APC]), have greatly increased the repertoire of fluorochromes available for use in immunofluorescence assays. These tandems are useful because of their high extinction coefficient, high quantum efficiency, and their emission spectrum that extends into the far red.

In this article, we describe the application of the originally described RET tandem (1), in which PE is covalently

linked to APC (PE-APC; Fig. 1). Newly developed chemistries and conjugation techniques have made PE-APC considerably easier to use as a tag for monoclonal antibodies or streptavidin (SA), thereby making PE-APC easily accessible for flow cytometric immunophenotyping.

PE-APC has the excitation spectrum of both PE and APC (and is well excited by the 488-nm argon laser line), but an emission spectrum that is primarily that of APC. By varying the stoichiometry of APC to PE, we synthesized fluorophores with very little PE emission and substantial APC emission, making it easily distinguishable from PE in simultaneous multicolor immunofluorescence applications.

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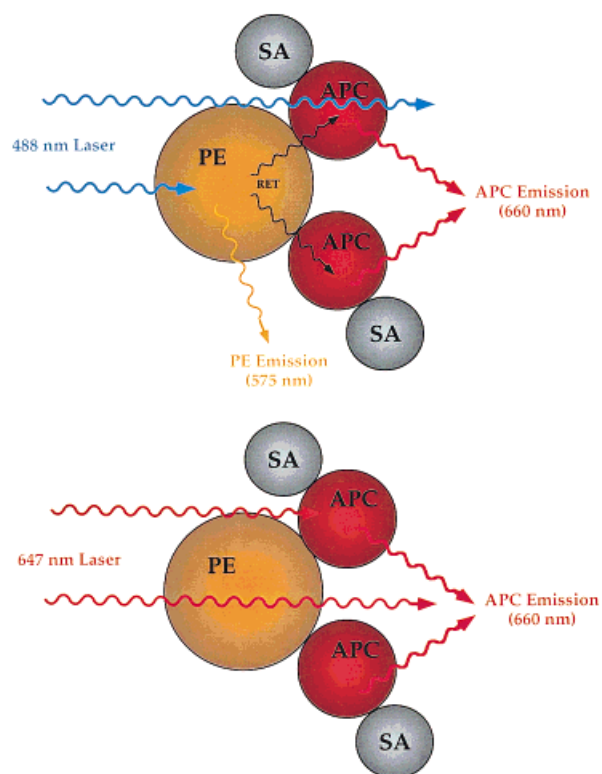


FIG. 1. Schematic representation of PE-APC RET. **Top:** PE-APC dyes are covalent complexes of PE and APC that can be conjugated to monoclonal antibodies, or, as shown here, to SA. In close proximity, PE and APC can become a RET pair, in which PE is the donor and APC the acceptor. When PE is excited (e.g., by 488 nm laser light), its energy can be emitted as a photon (orange emission, ~ 575 nm) or it can be transferred to APC. The excited APC can then emit the energy with the characteristic emission spectrum of APC (red emission, ~ 660 nm). The ideal RET dye transfers 100% of its energy from the donor to the acceptor, with no donor fluorescence emission. The stoichiometric ratio of APC:PE will determine the transfer efficiency, and thus the relative quenching of the PE emission: higher ratios of APC:PE will show greater transfer efficiency and greater quenching. **Bottom:** The PE-APC complex can also be excited by red laser light (e.g., 647 nm). Because the PE moiety cannot be excited, the only fluorescence occurs when the APC is excited and emits with its characteristic spectrum.

The excitation and emission spectra of PE-APC are very similar to those of Cy5PE and might provide an alternative to Cy5PE in immunofluorescence experiments. We compare several properties of Cy5PE and PE-APC conjugates and find that the PE-APC tandem should prove to be a viable substitute for Cy5PE in most fluorescence applications.

MATERIALS AND METHODS

Antibody Reagents

Unconjugated antibodies were purified from ascites or from tissue culture supernatant or they were obtained in concentrated form from PharMingen (San Diego, CA). Conjugated antibodies were prepared using the method described by Roederer (2).

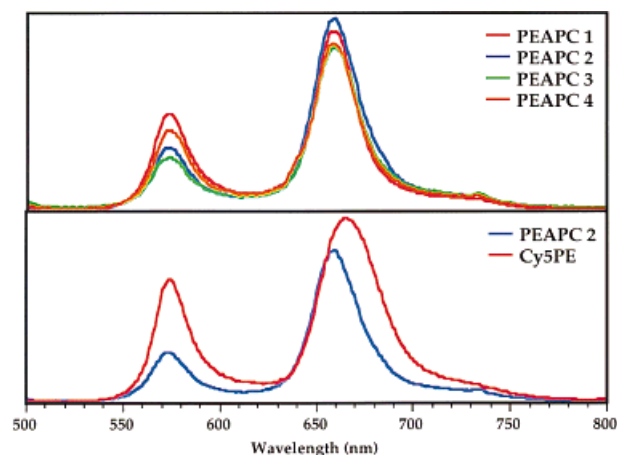


FIG. 2. Fluorescence spectra of different PE-APC preparations. **Top:** The fluorescence emission spectra (488 nm excitation) of four different PE-APC preparations with different ratios of APC:PE are shown. PE-APC dyes 1, 2, and 3 were made using PE from the same species of *Porphyridium* algae. PE-APC dye 4 was made from a PE isolated from a different strain of red algae. PE-APC dyes 1, 2, and 3 have successively greater ratios of APC:PE in the fluor complex. **Bottom:** Comparison of the emission spectrum of PE-APC 2 with Cy5PE. Cy5PE is a commonly used RET dye where PE is the donor and Cy5 the acceptor. The spectra are quite similar, with the exception that the PE-APC has a narrower red emission band.

Synthesis of PE-APC-SA Complexes

B-PE and APC were isolated from algae grown commercially by ProZyme (San Leandro, CA). The four lots of PE-APC tested in this study were synthesized similarly, having only different ratios of PE to APC in the tandem generation step. Briefly, PE was dialyzed into the "A" buffer (50 mM sodium phosphate, 1 mM EDTA, pH 7.0). The concentration of PE was adjusted to 5 mg/ml. SMCC (Pierce, Rockford, IL) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/ml; 0.54 mg per milligram of PE was added to the PE and incubated for 1 h. The SMCC-activated PE was dialyzed against the "PBP" buffer (50 mM MES, 2 mM EDTA, pH 6.0) and concentrated to 10 mg/ml using a centrifugation kit (Amicon, Danvers, MA).

APC was exchanged over a desalting column into the "IT" buffer (50 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, pH 8.0). 2-iminothiolane (Pierce) was dissolved in DMSO at 5 mg/ml; 0.38 mg per milligram of APC was added to the APC solution and incubated for 45 min. The thiolated APC was exchanged over a desalting column into the PBP buffer.

The activated PE and APCs were mixed at various molar ratios and incubated for 60 min. The tandems were purified as the leading peak on a Biogel A0.5M column equilibrated with the A buffer. The fractions containing the tandem were pooled and concentrated to 1 ml and reacted again with SMCC for 60 min. This complex was again exchanged into the PBP buffer on a desalting column.

Five milligrams of SA (ProZyme) was dissolved in 0.22 ml of the IT buffer; 70 μ g of 2-iminothiolane in DMSO was added and the mixture incubated for 45 min. The iminothiolated SA was exchanged into the PBP buffer on a desalting column.

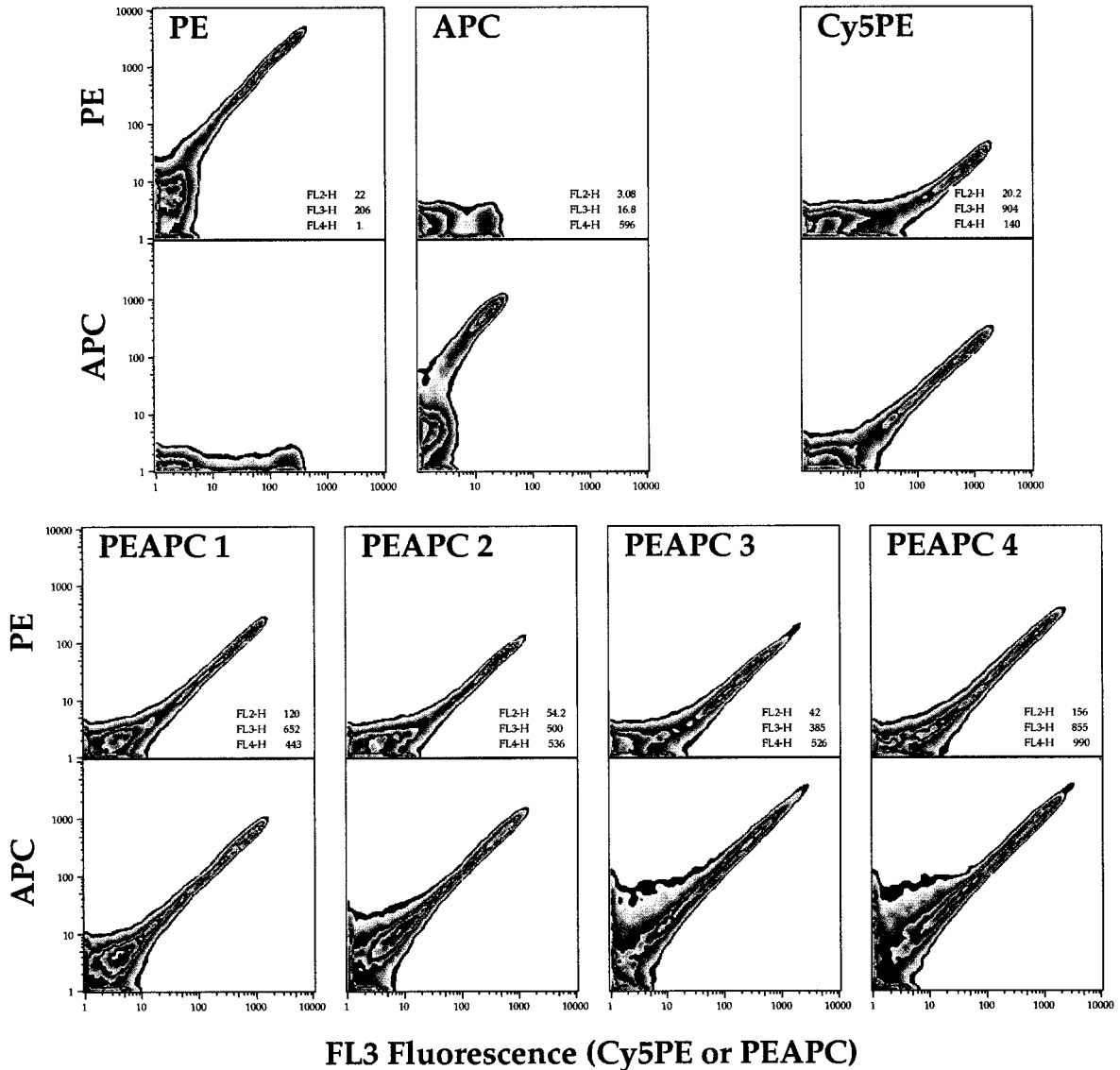


Fig. 3. Flow cytometric analysis of PE, APC, Cy5PE, and PE-APC dyes. Human PBMC were stained with biotinylated anti-CD8, washed, and split into equal aliquots. Aliquots were stained with saturating levels of SA-conjugated dyes. Single-stained samples were analyzed on a FACSCalibur. For each dye, bivariate density plots of the FL2 (PE) or FL4 (APC) channel versus the FL3 (Cy5PE/PE-APC) channel are shown. Compensation settings were set to 0 to illustrate the spectral overlap. The numbers in the box show the median scale fluorescence for each channel. The data from this experiment are further analyzed in Figure 4. Note that the PE-APC dyes show similar fluorescence intensity as Cy5PE, with slightly greater spectral overlap into the PE channel.

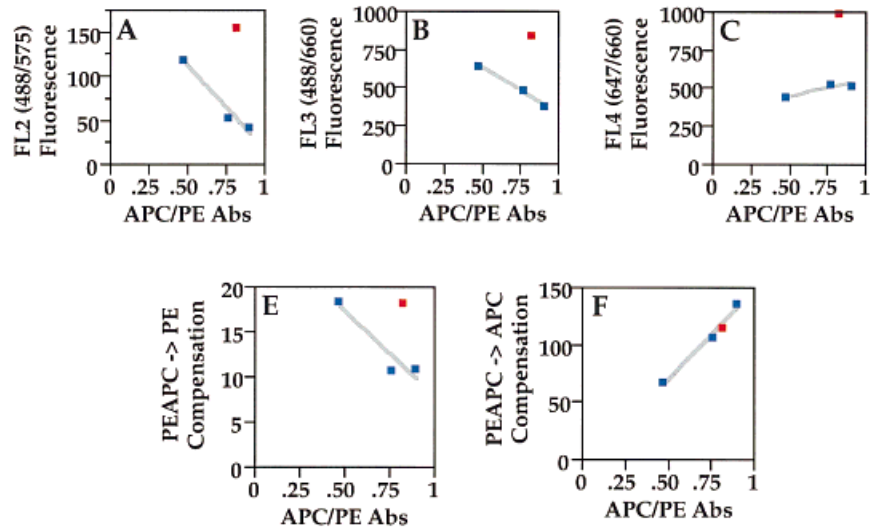
Finally, the activated SA and the PE-APC complexes were mixed at different ratios and incubated for 60 min. Twenty-five microliters of 10 mg/ml N-ethylmaleimide (Pierce) in DMSO was added to oxidize all free sulfhydryls and the reaction was incubated for 20 min. The final product was purified on a Biogel A0.5M column equilibrated with a storage buffer (10 mM Tris, 150 mM NaCl, pH 8.2) to remove unincorporated SA.

Cell Staining and Flow Cytometric Analyses

Human PBMC were prepared from heparanized whole blood by Ficoll density centrifugation. Washed cells were resuspended in RPMI supplemented with 4% fetal calf

serum. At least 10^6 cells were used for each stain. Cells were stained on ice for 15 min with fluorescently conjugated antibodies and washed three times with staining medium (biotin, flavin-deficient RPMI supplemented with 4% newborn calf serum and 0.02% sodium azide). For SA staining, cells were further stained with saturating concentrations of the SA conjugate on ice, followed by three additional washes. Data were analyzed on either a FACScan or FACSCalibur (Becton Dickinson, San Jose, CA) using Desk (3) or CellQuest (Becton Dickinson) for acquisition, and compensated and analyzed using FlowJo (Tree Star, San Carlos, CA). For identification of B cells and monocytes, peripheral blood mononuclear cells (PBMC) were

Fig. 4. The spectral properties of PE-APC (as measured by the flow cytometer). In these graphs, the red square represents PE-APC 4, which was synthesized using PE from a different species than PE-APCs 1-3. The lines are linear least-squares fits to the three data points from PE-APC 1-3. Data points are the median fluorescence of the CD8⁺ population as shown in Figure 3. **A-C**: The fluorescence intensity in the FL2 (PE) channel (A), the FL3 (PE-APC) channel (B), and the FL4 (APC) channel (C) is shown as a function of the APC:PE absorbance ratio. This ratio is proportional to the average ratio of APC to PE molecules in the complexes. **D,E**: Amount of compensation required to correct the spillover from the PE-APC RET fluorescence (FL3) into the PE channel (D) or the APC channel (E). In general, RET dyes are optimal when the RET fluorescence (B) is maximized and the compensations (D,E) are minimized.



stained with fluorescein-conjugated anti-CD19 and anti-CD14, respectively. For the three- and four-color experiments (see Figs. 6 and 7), we used fluorescent conjugates prepared in our laboratory of the antibodies shown.

Spectroscopy

Fluorescence emission and excitation spectra were obtained on an Aminco Bowman Series 2 spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD). Spectra were uncorrected for detector sensitivity. Absorbance spectra were collected on a Shimadzu UV160U spectrophotometer.

RESULTS

PE-APC complexes containing different ratios of PE:APC were synthesized and covalently linked to SA for use as counterstains for cells incubated with biotinylated antibodies. Using a 488-nm excitation, the emission spectra of the four PE-APC conjugates (Fig. 2) show a strong emission band at 660 nm. This is indicative of efficient RET

from the donor PE to the acceptor APC. In addition, there is a small peak at 575, due to incomplete RET. Note that the PE-APCs have similar emission properties to those of Cy5PE (Fig. 2), suggesting that they may be a useful alternative to Cy5PE in immunofluorescence applications.

The utility of a fluorochrome in flow cytometric experiments depends on several factors: the ease of conjugation to SA or monoclonal antibodies, the relative brightness (which itself depends on the quantum efficiency of the fluorochrome, the number of fluorochromes per SA or antibody, the detection efficiency in the emission region of the spectrum, and the autofluorescence of cells in that same region), and the spillover of fluorescence into other detectors (i.e., the compensation requirement). In general, better fluorochromes have a greater relative brightness and a lower spillover. The relative brightness and the compensation requirement can only be evaluated by staining cells and analyzing them on a suitable cytometer. Spectra (Fig. 2) can only provide a rough indication of their utility on flow cytometers.

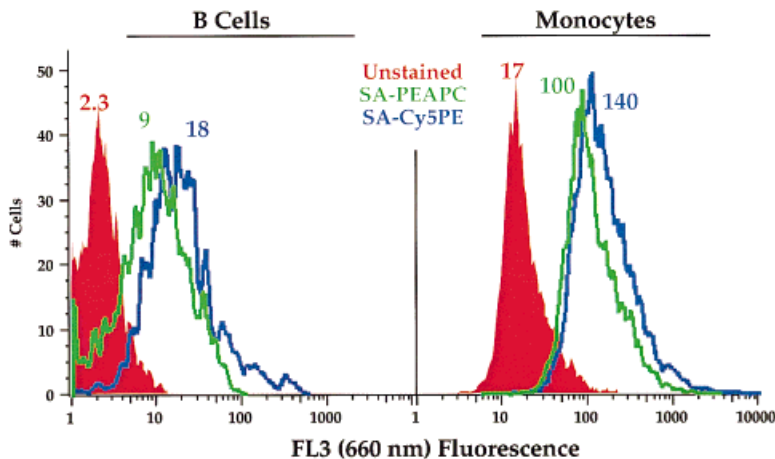


Fig. 5. Nonspecific binding of PE-APC and Cy5PE dyes. PBMC were stained with FITC CD19 (left) or FITC CD14 (right). One aliquot was further stained with saturating concentrations of Cy5PE-SA, one with saturating concentrations of PE-APC-SA, and one was left alone. Without a biotinylated antibody, there should be no SA binding on the cells. The graphs show the FL3 (Cy5PE or PE-APC) fluorescence for the cells that were not further stained (Unstained) or were incubated with Cy5PE-SA or PE-APC-SA. Numbers above the histograms show the median fluorescence in the FL3 channel of the gated B cells (CD19⁺ lymphocytes) or monocytes (CD14⁺ PBMC). On B cells, PE-APC shows 50% less background binding than Cy5PE. On monocytes, PE-APC shows 30% less background binding than Cy5PE.

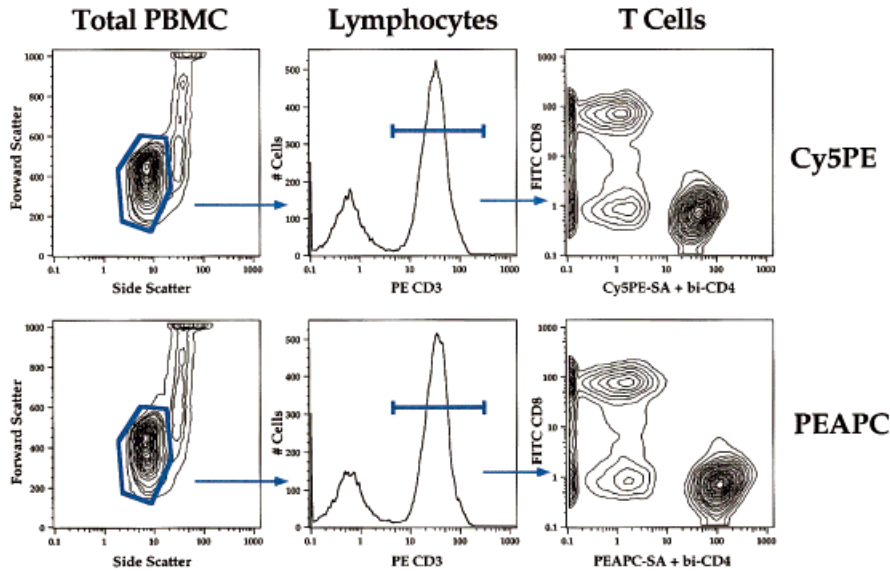


FIG. 6. Use of PE-APC in three-color flow cytometry. Human PBMC were stained with FITC-CD8, PE-CD3, Bi-CD4, washed, and then stained with either Cy5PE-SA or PE-APC-SA. Data were collected un-compensated. Singly stained compensation controls were used to adjust compensation using FlowJo. Gates are identical between the two samples. In this three-color experiment, the CD4 cells had similar brightness whether stained with PE-APC-SA or with Cy5PE-SA. Data were collected on a three-color FACScan.

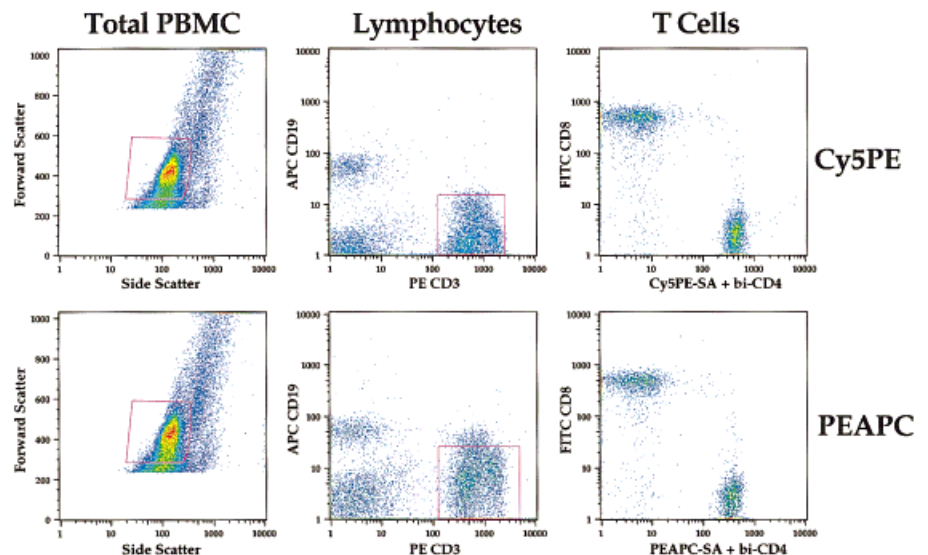
We stained cells with biotinylated anti-human CD8 and counterstained them with SA conjugates of PE, APC, Cy5PE, or the various PE-APC preparations (Fig. 3). The median fluorescence of the CD8+ T cells was compared with the median of the negative lymphocytes (selected by forward and side scatter gating). Based on the known percentage of CD8 T cells in the PBMC, we calculated the median of the CD8+ T cells without gating by simply calculating the appropriate percentile of the fluorescence distribution. For example, when CD8 T cells comprise 12% of the population, the 94th percentile of the fluorescence distribution corresponds to the median of the brightest 12% of the events (CD8 T cells). Note that the precise percentile used is irrelevant for the determinations of brightness and spillover, as long as the same percentile is used for all tubes collected from the same PBMC sample. Using these data, we can

evaluate the relative brightness of each conjugate as well as the compensation requirements (Fig. 4).

The PE-APC tandems behave as would be predicted for RET pairs (Fig. 4). For example, as the molar ratio of APC to PE increases, the efficiency of transfer also increases (as is shown by a decline in the PE fluorescence [Fig. 4A]). The increase in transfer efficiency directly translates into lower compensation requirements between the primary measurement channel (APC emission from the first laser, “FL3”) and the channel devoted to PE emission. On the other hand, as the ratio of APC:PE increases, the amount of direct APC fluorescence (APC emission from the second laser, “FL4”) increases, requiring more compensation between FL3 and FL4.

Thus, as is the case for Cy5PE, the optimal PE-APC dye may be different depending on the application. For three-

FIG. 7. Use of PE-APC in four-color flow cytometry. Human PBMC were stained with FITC CD8, PE CD3, Bi CD4, and APC CD19, washed, and then stained with either Cy5PE-SA or PE-APC-SA. Data were collected un-compensated. Singly stained compensation controls were used to adjust compensation using FlowJo. Gates are identical between the two samples. In this four-color experiment, the CD4 cells have similar CD4 fluorescence intensity whether revealed by Cy5PE or by PE-APC. Data were collected on a four-color FACSCalibur.



color applications, for which the direct APC fluorescence is irrelevant, the optimal PE-APC will be that with the greatest APC:PE ratio (with the greatest quenching of PE and with the greatest FL3 fluorescence). For applications utilizing APC in addition to PE-APC, the optimal dye will be one with a lower APC:PE ratio (having less quenching of PE, but also lower compensation requirements with the FL4 channel).

For the experiments shown in Figures 5–7, we selected PE-APC 2. PE-APC 2 has the intermediate APC:PE ratio of the three PE-APC dyes, a compromise to be used for experiments in both three-color and four-color settings.

One of the disadvantages of Cy5PE conjugates is that they display unusually high background binding on selected subsets (i.e., B cells and monocytes). In order to evaluate the background binding of these reagents to B cells and monocytes, we stained PBMC with fluorescein conjugates of CD19 and CD14, respectively, together with the PE-APC-SA or Cy5PE-SA. Because there were no biotinylated antibodies in the staining, any PE-APC or Cy5PE staining is due to nonspecific binding of the fluorochrome complexes to the cells.

Although the PE-APC tandems also seem to show selectively increased binding to B cells and monocytes, it is only 50–70% that of Cy5PE (Fig. 5). This may indicate that the mode of this nonspecific binding is not due to the presence of Cy5 on PE (as previously thought). Rather, it is due to a change induced in the PE molecule after the chemistries used for conjugation to heterologous molecules, revealing or creating a binding site for some molecule(s) found on B cells and monocytes.

We evaluated the use of PE-APC in three-color (Fig. 6) and four-color (Fig. 7) immunofluorescence analysis by flow cytometry. For these experiments, cells were stained with fluorescent conjugates of antibodies against CD3, CD8, and (for the four-color) CD19, together with biotinylated anti-CD4. The cells were then split into two aliquots and counterstained with either PE-APC-SA or Cy5PE-SA.

Figure 6 demonstrates that in three-color analysis, after appropriate compensation, there is no difference in the fluorescein or PE channels. The FL3 channel (PE-APC or Cy5PE) is directly comparable (in this case, the PE-APC was brighter than Cy5PE). On a different (four-color) instrument, a similar experiment was performed with the addition of a fourth color (anti-CD19 conjugated to APC). Again, there were no discernible differences in the fluorescence distributions of the other conjugates after appropriate compensation. The PE-APC and Cy5PE reagents showed nearly identical fluorescence distributions. Together, Figures 6 and 7 show that typical three- and four-color analyses can easily use PE-APC in place of Cy5PE with no untoward effects.

DISCUSSION

PE-APC dyes (RET dyes comprising covalently linked PE and APC) have been synthesized. These dyes show the expected fluorescence properties of RET pairs: excitation spectrum with components of both dyes and an emission spectrum principally of the acceptor (APC).

For multicolor immunofluorescence applications, PE-APC would be used in place of Cy5PE. Therefore, we compared PE-APC with Cy5PE directly in flow cytometric analysis of PBMC staining. In most regards, the PE-APC performed identically to Cy5PE, having roughly the same relative brightness and similar compensation requirements (Fig. 3). PE-APC showed less background on B cells and monocytes, although still displaying a significant non-specific binding.

In applications where the direct APC emission is not being measured, in which the spillover from the PE-APC channel into the APC channel is irrelevant, a PE-APC preparation such as PE-APC 4 in these experiments can be used. This complex showed much brighter immunofluorescence staining than with Cy5PE staining.

PE-APC will have significantly lower compensation requirements in experiments that simultaneously use other PE tandems such as Cy5.5PerCP, Cy5.5PE, and Cy7PE. This is because the emission spectrum of APC is shifted to the blue and is more narrow than that of Cy5. Therefore, filter sets that better separate the specific emissions of Cy5.5PE (and Cy7PE) from PE-APC than from Cy5PE can be used. This can be a significant advantage when using Cy5.5PE because the spillover between Cy5PE and Cy5.5PE is typically 60–70%. The spillover between PE-APC and Cy5.5PE can be as low as 6%. Note that this advantage in compensation does not depend on the specific PE-APC or Cy5PE preparation used because the spillover from the APC or Cy5 into the Cy5.5 or Cy7 channels is a property only of the APC or Cy5 moieties. Therefore, the ratio of APC (or Cy5) to PE has no effect on the compensation requirement between FL3 and the Cy5.5 emission detector.

In conclusion, PE-APC is a useful fluorochrome for immunofluorescence staining applications, particularly flow cytometry. As with any RET tandem, it can be made at different donor:acceptor ratios that provide alternatively brighter emission or lower compensation requirements; the optimal conjugate may differ from application to application. However, considering how bright this reagent is, the choice of one with low compensation requirements (as has been done for commercial Cy5PE conjugates) will prove useful in most applications.

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