

# Detection of Endogenous and Antibody-Conjugated Alkaline Phosphatase With ELF-97 Phosphate in Multicolor Flow Cytometry Applications

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**Background:** The fluorogenic alkaline phosphatase (AP) substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF<sup>®</sup>-97 phosphate, for *Enzyme-Labeled Fluorescence*) has been used primarily in microscope-based imaging applications to detect endogenous AP activity, antigens and various ligands in cells and tissues, and nucleic acid hybridization. In a previous study, we demonstrated the applicability of ELF-97 phosphate for detecting endogenous AP activity by flow cytometry. In this study, we show that the spectral characteristics and high signal-to-noise ratio provided by the ELF-97 phosphate make it a useful label for immunodetection via flow cytometry. It can be combined with a variety of other fluorochromes for multiparametric flow cytometry analysis of both endogenous AP activity and intracellular and extracellular immunolabeling with AP-conjugated antibodies.

**Methods:** ELF-97 phosphate detection of endogenous AP activity in UMR-106 rat osteosarcoma cells was combined with intracellular antigen detection using Oregon Green 488 dye-conjugated secondary antibodies and DNA content analysis using propidium iodide (PI) or 7-aminoactinomycin D (7-AAD). ELF-97 phosphate detection of endogenous AP was also tested for spectral compatibility with a variety of other commonly used fluorochromes. ELF-97 phosphate was then used to directly label intracellular antigens via AP-conjugated antibodies, again combined with the analysis of DNA content using PI and 7-AAD. ELF-97 phosphate was also used to directly detect extracellular antigens. It was combined with Oregon Green 488 dye, phycoerythrin (PE), and PE-Cy5 dye-labeled antibodies for simultaneous four-color analysis. All

samples were analyzed on a dual-beam flow cytometer, with UV excitation of the ELF-97 alcohol reaction product.

**Results:** Application of the ELF-97 phosphate to detect AP was found to be compatible with immunodetection and DNA staining techniques. It was also spectrally compatible with a variety of other fluorochromes. Endogenous AP activity could be detected simultaneously with both intracellular antigens labeled using Oregon Green 488 dye, PE, Cy5 dye and Alexa Fluor 568 dye-conjugated antibodies, and DNA content analysis with PI or 7-AAD. This multiparametric assay accurately delineated the distribution of AP in cycling cells and was able to identify cell subsets with varying endogenous AP levels. The ELF-97 alcohol reaction product was found to be an effective label for intracellular antigen immunolabeling with AP-conjugated reagents, and could also be combined with PI and 7-AAD. ELF-97 phosphate was also found to be a useful label for extracellular antigen immunolabeling with AP conjugates, and was compatible with Oregon Green 488 dye, PE, and PE-Cy5 dye-labeled antibodies for four-color surface labeling with minimal spectral overlap and color compensation.

**Conclusions:** ELF-97 phosphate was shown to be a useful label for both endogenous and antibody-conjugated AP activity as detected by flow cytometry. Its spectral characteristics allow it to be combined with a variety of fluorochromes for multiparametric analysis. *Cytometry* 43: 117-125, 2001. Published 2001 Wiley-Liss, Inc.<sup>†</sup>

**Key terms:** ELF-97; alkaline phosphatase; flow cytometry; multicolor analysis

The development of fluorogenic alkaline phosphatase (AP) substrates has been important to the advent of sensitive assays for AP activity, which serves as an endogenous marker of cell phenotype and as a reporter when conjugated to antibodies and nucleic acid probes (1,2). In general, these fluorogenic enzyme substrates are essentially nonfluorescent when their phosphate group is attached but fluoresce brightly upon cleavage in the pres-

ence of the appropriate mercury-arc lamp or laser excitation. Fluorescein-, coumarin-, and other fluoro-

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phore-based substrates have been useful in solution-based microplate and gel-based AP detection systems, but have proven to be less valuable for localizing AP activity in fixed intact cells (2-6). These compounds are often water soluble both prior to and upon cleavage, resulting in poor cellular retention of the cleaved substrate (1,2).

Singer et al. (7) described the development of the fluorogenic AP substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF<sup>®</sup>-97 phosphate for *Enzyme-Labeled Fluorescence*). Although this substrate itself is water soluble and essentially nonfluorescent, hydrolysis by AP yields a ultraviolet (UV)-excited, highly fluorescent yellow-green alcohol precipitate. The ELF-97 phosphate has been particularly useful for cell and tissue labeling applications, both for the detection of endogenous AP and AP-conjugated immunocomplexes, for which retention of the insoluble cleavage product is critical to the localization of the AP activity. The use of the ELF-97 phosphate has been demonstrated in several histochemical applications including localization of endogenous AP activity, detection of AP-conjugated antibodies and cytological probes, and localization of AP-transfected cells in regenerating newt limbs (5,8-11). ELF-97 phosphate has also been used to detect AP-conjugated nucleic acid probes hybridized to chromosomes, mRNAs in intact cells and tissue sections, and DNA in gels and on filters (12,13).

Although the ELF-97 phosphate has been used primarily in histochemical applications, its UV excitation peak suggests that it would also be efficiently excited by the UV lines obtained from argon- and krypton-ion lasers commonly found on flow cytometers. In fact, two recent reports (14,15) described methods for using ELF-97 phosphate to measure endogenous AP activity in marine phytoplankton and in avian and mammalian cells by flow cytometry. The ELF-97 phosphatase substrate is a potentially important flow cytometric reagent for several reasons. First, no other good fluorogenic assays for AP currently exist that work well in intact cells and under the excitation/emission conditions commonly found on flow cytometers. Fast Red and Fast Red Violet azo dye assays have been described for both endogenous and AP-conjugated antibody detection by flow cytometry, but these assays are hampered by the high intrinsic fluorescence of the unreacted azo dye (16-21). Even under optimal excitation conditions (530 nm, an unusual laser wavelength not found on many cytometers), the Fast Red Violet azo dye adduct product formed by AP activity accounts for only 80% of the total cellular fluorescence (15; Telford and Doty, unpublished data). In contrast, the ELF-97 alcohol generated by AP activity accounts for 98% of the total cellular fluorescence in AP-expressing UMR-106 cells as detected by flow cytometry (15).

The second reason for the potential importance of ELF-97 phosphate as a labeling reagent for use in flow cytometry is the shortage of useful UV-excited fluorochromes applicable to flow cytometry. UV excitation causes considerable autofluorescence in cells in the 400-480 nm range. As a result, UV-excited fluorochromes often

have low signal-to-noise ratios, limiting their usefulness for cellular labeling. Although several coumarin-based fluorophores (most commonly 7-aminomethylcoumarin) have been used for flow cytometry, their emission peaks fall within the range of maximum autofluorescence, limiting their usefulness to labeling only the densest antigens. Coumarin-based fluorochromes are also easily photobleached, further limiting their usefulness for flow cytometry. ELF-97 phosphate, in contrast, is extremely bright and photostable, with widely separated excitation and emission peaks and an emission maximum in the fluorescein range. This combined brightness, photostability, and large Stokes shift makes ELF-97 phosphate a promising candidate for extracellular and intracellular labeling applications. UV lasers are common fixtures on multibeam flow cytometers; a bright, photostable, UV-excited molecule would therefore be a very useful addition to the collection of available phenotyping fluorochromes.

We have previously demonstrated that the ELF-97 phosphate provides sensitive detection of endogenous AP activity by flow cytometry (15). In this study, we further evaluate its usefulness as a flow cytometric fluorochrome by using it to detect endogenous AP activity in combination with other cell cycle parameters, and intracellular and extracellular antigen expression using AP-conjugated labeling reagents. We demonstrate both the usefulness of ELF-97 phosphate as a fluorescent phenotyping reagent and its compatibility with several other commonly used fluorochromes.

## MATERIALS AND METHODS

### Cell Culture and Fixation

UMR-106 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and trypsinized prior to passage or harvest. EL-4 murine thymoma cells (American Type Culture Collection) were maintained in RPMI with 10% FBS and passed every 3 days. Mouse thymocytes and splenocytes were obtained from euthanized young (6-20-week-old) male BALB/c mice maintained in the National Cancer Institute's barrier animal facility. Thymuses and spleens were removed and forced through sterile nylon mesh to obtain single cell suspensions. Prior to labeling, splenocytes were depleted of erythrocytes over Ficoll-Hypaque gradients.

For endogenous and intracellular AP detection, cells were treated with 70% EtOH at 4°C for up to 24 h and washed with phosphate-buffered saline (PBS) prior to ELF-97 phosphate treatment. For extracellular AP detection, cells were immunophenotyped in the viable state prior to ELF-97 phosphate treatment.

### Detection of Endogenous AP With Simultaneous Intracellular Antigen and/or DNA Content Analysis

Ethanol-treated UMR-106 cells were washed twice by centrifugation with PBS containing 1% bovine serum albumin (BSA), 0.01% Tween 20, and 0.1% sodium azide (termed intracellular label buffer) to remove ethanol. Cells

were then labeled with monoclonal antibodies against proliferating cell nuclear antigen at 10  $\mu\text{g/ml}$  (PCNA, clone PC10, Sigma, St. Louis, MO) in 200  $\mu\text{l}$  intracellular label buffer overnight at 4°C. Cells were subsequently washed twice with intracellular label buffer and labeled with AP-conjugated anti-mouse IgG at 25  $\mu\text{g/ml}$  (Jackson ImmunoResearch, West Grove, PA) for 4 h at 4°C and washed twice with intracellular label buffer. Cells were then washed once with the ELF-97 developing buffer provided by the manufacturer and incubated with ELF-97 phosphate according to the manufacturer's directions (ELF-97 endogenous phosphatase detection kit, Molecular Probes, Eugene, OR). Briefly, the ELF-97 phosphate solution was filtered through a 0.2- $\mu\text{m}$  centrifuge filter to remove precipitated reaction product. The ELF-97 phosphate was then added to the cells in a final volume of 100  $\mu\text{l}$  to provide a 1:20 final dilution of the provided stock solution. Cells were then incubated in the reaction solution at room temperature for 30 min. The reaction was stopped with levamisole at 1 mM in PBS with no washing. Cells were subsequently washed with PBS alone and stained with 7-aminoactinomycin D (7-AAD; Sigma) at 10  $\mu\text{g/ml}$  (no RNase) in PBS and analyzed by flow cytometry. For some experiments, the distribution of AP expression in different cell cycle phases was compared by gating cells at a particular DNA content fluorescence and calculating the mean fluorescence intensity (MFI) ratio between cells labeled with ELF-97 phosphate in the absence or presence of the AP inhibitor, levamisole. The resulting values were then plotted as AP expression ratio versus DNA content.

#### **Detection of AP-Conjugated Antibodies Against Intracellular Antigens With Simultaneous DNA Content Analysis**

Ethanol-treated EL-4 thymoma cells were washed twice by centrifugation with intracellular label buffer to remove ethanol. Cells were subsequently labeled overnight at 4°C with monoclonal antibodies against either cyclin B1 or PCNA, as described above. Cells were labeled with AP-conjugated anti-mouse IgG at 25  $\mu\text{g/ml}$  in a 200- $\mu\text{l}$  sample volume (Jackson ImmunoResearch, West Grove, PA) for 4 h at 4°C and washed twice with intracellular label buffer. Cells were then washed with the ELF-97 wash buffer provided by the manufacturer and labeled with ELF-97 phosphate (ELF-97 cytological labeling kit) as described above with modifications for protein-tagged AP detection according to the manufacturer's directions. Cells were then washed once with PBS and stained with propidium iodide (PI) as described above. For comparison, some samples were labeled with Oregon Green 488 dye-anti-mouse IgG (25  $\mu\text{g/ml}$ ) and stained with the UV-excited DNA dye Hoechst 33258 at 1  $\mu\text{g/ml}$  in PBS (Fluka, Milwaukee, WI) with no RNase. EL-4 cell controls incubated without AP-conjugated anti-mouse IgG and stained with the ELF-97 phosphate showed no detectable fluorescence above background.

#### **Detection of AP-Conjugated Antibodies Against Extracellular Antigens With Simultaneous Multicolor Extracellular Antigen Detection**

Viable mouse thymocytes or splenocytes were washed in PBS containing 2% FBS and 0.01% sodium azide (termed extracellular label buffer) and labeled in a 200- $\mu\text{l}$  sample volume with one of the following biotin-conjugated antibodies for 30 min at 4°C: anti-mouse CD8 (10  $\mu\text{g/ml}$ ; in-house preparation), anti-mouse Thy1.2 (10  $\mu\text{g/ml}$ ; Becton-Dickinson Immunocytometry Systems, San Jose, CA), or anti-mouse CD95/Fas (5  $\mu\text{g/ml}$ ; Pharmingen, San Diego, CA). Cells were then washed with extracellular label buffer and labeled with AP-streptavidin (25  $\mu\text{g/ml}$ ; Molecular Probes). For comparison purposes, some cells were alternatively labeled with fluorescein isothiocyanate (FITC)-streptavidin (Jackson ImmunoResearch), Oregon Green 488 dye-streptavidin (Molecular Probes), and Cy5 dye-streptavidin (Caltag, Burlingame, CA) at 50, 25, and 25  $\mu\text{g/ml}$ , respectively, followed by washing and analysis.

For multicolor experiments, AP-labeled cells were subsequently labeled with combinations of the following antibodies as above: carboxyfluorescein (CFL)-anti-mouse CD8 (25  $\mu\text{g/ml}$ ; in-house preparation and conjugation), phycoerythrin (PE)-anti-mouse CD95/Fas (25  $\mu\text{g/ml}$ ; Pharmingen), and PE-Cy5 dye-anti-mouse CD4 (25  $\mu\text{g/ml}$ ; Caltag). Following immunolabeling and washing, cells were incubated with ELF-97 phosphate (ELF-97 cytological detection kit) using the procedure described above with modifications as described by the manufacturer (Molecular Probes). Cells were then resuspended in PBS with 0.1% sodium azide and analyzed by flow cytometry within 2 h of labeling. As for EL-4 cells above, murine thymocyte and splenocyte controls incubated without the AP-streptavidin conjugate and stained with the ELF-97 phosphate showed no detectable fluorescence above background levels.

#### **Flow Cytometry**

Cells were analyzed on a Becton-Dickinson FACSVantage SE equipped with a Coherent I-90 water-cooled argon-ion (primary position) and a Coherent I-302 water-cooled krypton-ion and Spectra-Physics helium-neon lasers (secondary position). In all cases, forward and side scatter were detected with the primary laser (emitting at either 488 or 351 nm). For detection of endogenous AP in UMR-106 cells with simultaneous intracellular antigen using Oregon Green 488 dye and DNA content analysis using 7-AAD, cells were excited using the primary argon-ion laser tuned to 488 nm. Oregon Green 488 dye was detected through a  $530 \pm 30\text{-nm}$  filter and 7-AAD fluorescence reflected off a 610 SP dichroic and detected through a  $675 \pm 20\text{-nm}$  filter. The ELF-97 alcohol reaction product was excited using the secondary krypton-ion laser tuned to 351 nm, with fluorescence detected through a  $535 \pm 45\text{-nm}$  filter. For endogenous AP detection with simultaneous Cy5 dye or Alexa Fluor 568 dye labeling, ELF-97 alcohol was excited using the primary argon-ion laser tuned to 335 nm, with detection through a

535  $\pm$  45-nm filter. Cy5 dye was excited with the secondary helium-neon laser emitting at 632 nm, with the signal detected through a 675  $\pm$  20-nm filter. Alexa Fluor 568 dye was excited by the secondary krypton-ion laser tuned to 568 nm, with detection through a 610  $\pm$  20-nm filter.

For detection of AP-conjugated antibodies against intracellular antigens in EL-4 cells with simultaneous DNA content analysis, PI was excited using the primary argon-ion laser tuned to 488 nm, with detection through a 585  $\pm$  22-nm filter. The ELF-97 alcohol reaction product was excited with the secondary krypton-ion laser tuned to 351 nm, with detection through a 535  $\pm$  45-nm filter as described above. For detection of AP-conjugated antibodies against extracellular antigens with simultaneous multicolor extracellular antigen detection, CFL, PE, and PE-Cy5 dye were excited using the primary argon-ion laser tuned to 488 nm. CFL was detected through a 530  $\pm$  30-nm filter, the PE signal was reflected off a 560 short pass dichroic and detected through a 575  $\pm$  22-nm filter, and the PE-Cy5 dye signal was reflected off a 610 short pass filter and detected through a 675  $\pm$  20-nm filter. The ELF-97 reaction product was excited with the secondary krypton-ion laser tuned to 351 nm, with detection through a 535  $\pm$  45-nm filter as described above.

## RESULTS

### Detection of Endogenous AP With Simultaneous Intracellular Antigen and DNA Content Analysis

We first examined the ability of ELF-97 phosphate to measure endogenous AP activity simultaneously with detection of intracellular antigen labeling and DNA content. UMR-106 cells were treated with ethanol, washed, and immunolabeled for PCNA using an unlabeled primary monoclonal antibody and an Oregon Green 488 dye-conjugated secondary as described in the Materials and Methods. The cells were subsequently labeled with ELF-97 phosphate to detect endogenous AP and stained with the DNA binding dye, 7-AAD. The cells were then analyzed by dual-laser flow cytometry, exciting Oregon Green and 7-AAD using an argon-ion laser emitting at 488 nm and ELF-97 using a krypton-ion laser emitting at 351 nm.

The results are shown in the three dimensional (3-D) plots in Figure 1. We found that endogenous AP, PCNA expression, and DNA content were successfully analyzed simultaneously with little fluorescence interference or overlap by any of the individual fluorescent components. PI was also spectrally compatible with ELF-97 phosphate and could be substituted for 7-AAD in these experiments, although the longer wavelength 7-AAD was preferable. Interestingly, simultaneous labeling for endogenous AP, PCNA, and DNA content revealed the presence of an AP-low subpopulation in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle that was also PCNA-low, with higher levels of AP in the S-phase and the G<sub>2</sub>/M phase. This phenomenon is visible in Figure 1a and in the 2-D plot in Figure 1e. Calculation of the MFI ratio between cells labeled with ELF-97 substrate and levamisole controls showed that the AP activity

in the G<sub>1</sub> phase of the cell cycle was indeed lower than that in the S- and G<sub>2</sub>/M phase (Fig. 1f). This low AP subset of the G<sub>1</sub> compartment also corresponded with decreased PCNA expression (Fig. 1g). This multiparametric assay accurately delineated the distribution of AP in cycling cells and was able to identify cell subsets with varying endogenous AP levels.

This assay also demonstrated that the ELF-97 alcohol and Oregon Green 488 dye signals could be detected simultaneously with no interlaser fluorescence compensation, despite the presence of a UV peak in the Oregon Green 488 dye excitation spectrum. Figure 2 illustrates the control samples labeled with either ELF-97 alcohol or Oregon Green 488 dye only versus DNA labeling, demonstrating their spectral compatibility. Samples treated with the AP inhibitor, levamisole, had no detectable ELF-97 alcohol. Simultaneous labeling with the Oregon Green dye, PI, or 7-AAD was also found to cause no loss in the ELF-97 alcohol signal; this was ultimately true for all fluorochromes evaluated for multicolor use with the ELF-97 phosphate. Intracellular immunolabeling and DNA content analysis were therefore found to be compatible with the fixation and assay conditions for the ELF-97 phosphatase substrate.

Other immunolabeling and DNA binding fluorochromes were also found to be compatible with detection of endogenous AP by the ELF-97 alcohol. Figure 3 shows simultaneous AP detection with ELF-97 phosphate and immunodetection of PCNA expression with the helium-neon laser (632 nm)-excited fluorochrome Cy5 (Fig. 3a) and the yellow krypton-ion laser (568 nm) excited Alexa Fluor 568 dye (a fluorochrome with an excitation/emission spectrum similar to that of lissamine rhodamine; Fig. 3b). In both cases, the immunolabeling fluorochrome and ELF-97 alcohol were excited with spatially separated lasers requiring minimal or no interlaser color compensation. The monomeric unsymmetrical cyanine nucleic acid dyes TO-PRO-3 (EX = 632 nm, EM = 670 nm) and BO-PRO-3 (EX = 568 nm, EM = 610 nm) were also compatible with the ELF-97 alcohol (data not shown).

### Detection of AP-Conjugated Antibodies Against Intracellular Antigens With Simultaneous DNA Content Analysis

Up to now, the flow cytometric applications of the ELF-97 phosphate substrate have been primarily limited to the detection of endogenous AP activity. As stated previously, the dearth of effective UV-excited immunolabeling fluorochromes makes ELF-97 phosphate a useful candidate for the detection of intracellular and extracellular antigens by flow cytometry. The low molecular weight and photostability of the ELF-97 phosphate substrate may make it particularly suitable for intracellular labeling applications. Comparisons were therefore made between intracellular immunolabeling based on ELF-97 phosphate and traditional fluorochromes.

This application is illustrated in Figure 4. Murine EL-4 thymoma cells were again chosen for these studies due to their negligible endogenous AP levels. Ethanol-

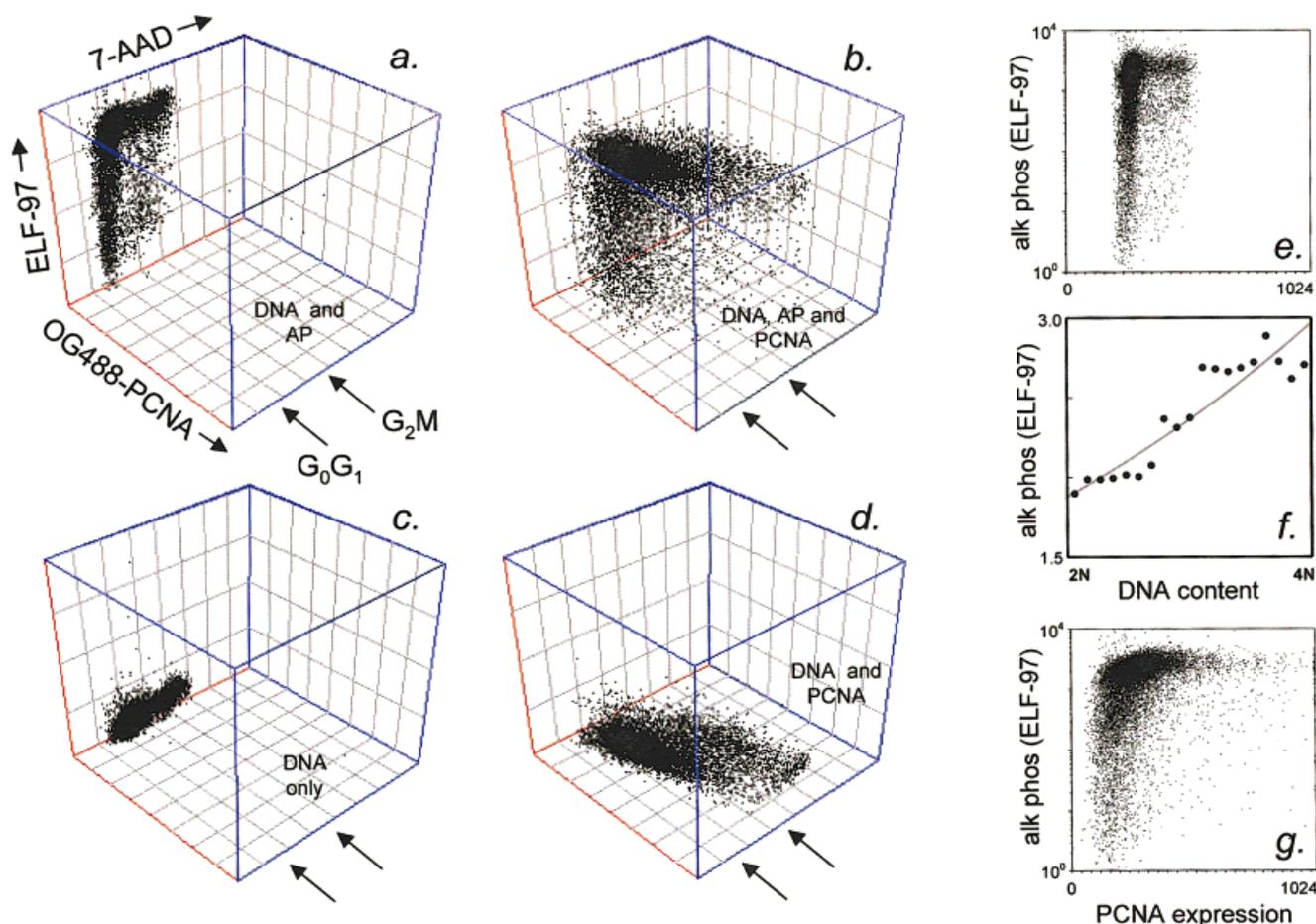


Fig. 1. Simultaneous analysis of Oregon Green 488 dye, 7-AAD, and ELF-97 phosphate fluorescence by flow cytometry. UMR-106 cells were immunolabeled for PCNA, detected with Oregon Green 488 dye-anti-mouse IgG secondary, labeled with ELF-97 phosphate to detect endogenous AP activity, and stained with 7-AAD for DNA content analysis. The cells were analyzed for all three fluorochromes by dual-beam flow cytometry as described in the Materials and Methods. One- and two-color controls were also prepared and analyzed. **a**: ELF-97 alcohol and 7-AAD labeling only (no Oregon Green 488 dye); **b**: all three fluorochromes; **c**: 7-AAD labeling only; **d**: Oregon Green 488 dye and 7-AAD labeling only (no ELF-97 alcohol); **e**: 2-D plot of DNA content versus endogenous AP activity (ELF-97 alcohol); **f**: AP expression (calculated as the ratio between MFI of ELF-97-labeled samples without and with levamisole treatment) over the 2N-4N DNA content distribution; **g**: 2-D plot of AP expression (ELF-97 alcohol) versus PCNA expression. ELF-97 alcohol fluorescence was analyzed on a log scale. Oregon Green dye, PCNA fluorescence, and 7-AAD DNA content were analyzed on a linear scale.

treated EL-4 cells were labeled with an unconjugated monoclonal antibody against the M-phase cyclin B1, followed by secondary labeling with AP-conjugated anti-mouse IgG. The cells were then labeled with ELF-97 phosphate, stained with PI, and analyzed by flow cytometry (Fig. 4a). For comparison purposes, concurrent samples were secondary labeled with Oregon Green 488 dye-anti-mouse IgG and stained with the DNA binding dye, Hoechst 33258 (Fig. 4b). As shown in Figures 4a and 4b, ELF-97 phosphate gave good detection of cyclin B1, comparable to or exceeding that of Oregon Green 488 dye. ELF-97 alcohol labeling demonstrated the same cell cycle phase specificity as Oregon Green 488 dye immunolabeling, with the highest level of cyclin B1 expression in the G<sub>2</sub>/M phase compartment. Control samples in which the AP-conjugate IgG was omitted showed no ELF-97 alcohol labeling.

#### Detection of AP-Conjugated Antibodies Against Extracellular Antigens With Simultaneous Multicolor Extracellular Antigen Detection

The attributes that make ELF-97 phosphate a useful intracellular label would presumably also apply to detection of extracellular antigens as well. Unlike intracellular labeling, however, the deposited precipitate might not remain sufficiently localized to the reaction site during cell-surface labeling to allow for good detection, particularly in multicolor labeling protocols with many wash steps. The ELF-97-labeling procedure would also have to be compatible with antibody labeling procedures.

To determine if this was the case, viable mouse thymocytes were labeled with biotin-conjugated antibodies against CD8, CD90/Thy1.2 (both intermediate to high-expressed antigens), or CD95/Fas (a relatively lower-ex-

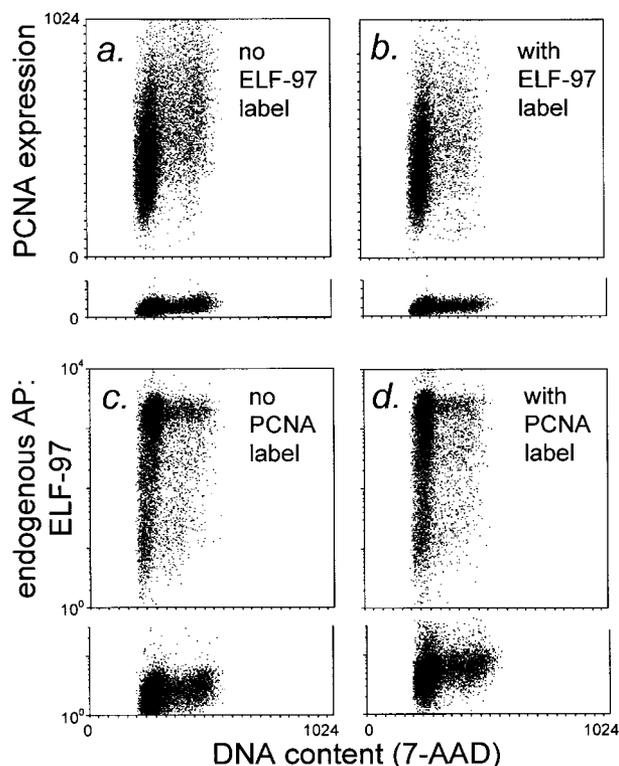


Fig. 2. Simultaneous analysis of Oregon Green 488 dye, 7-AAD, and ELF-97 alcohol fluorescence by flow cytometry. Two-parameter plots of the labeled cells described in Figure 1, illustrating Oregon Green 488 dye-PCNA expression in the absence or presence of ELF-97 alcohol labeling (a,b) and ELF-97 alcohol labeling in the absence or presence of Oregon Green 488 dye-PCNA labeling (c,d). Oregon Green 488 dye and ELF-97 alcohol therefore showed no spectral interference with one another. ELF-97 alcohol fluorescence was analyzed on a log scale. Oregon Green dye, PCNA fluorescence, and 7-AAD DNA content were analyzed on a linear scale.

pressed antigen) followed by AP-conjugated streptavidin secondary labeling and subsequent detection with ELF-97 phosphate labeling. For comparison purposes, some cells were then labeled with FITC-, Oregon Green 488 dye-, and Cy5 dye-conjugated streptavidin. The results are shown in Figure 5. ELF-97 phosphate successfully labeled CD8 and CD90, but the weakly expressed CD95 antigen was not detectable above background. Labeling for all antigens was found to be between FITC and Oregon Green 488 dye in overall fluorescent brightness. Control samples in which the AP-streptavidin was omitted showed no ELF-97 signal. Although not as bright as the relatively photostable Oregon Green 488 dye, in this application, the ELF-97 alcohol was found to be sufficiently sensitive to allow detection of antigens expressed at intermediate to high levels on cell surfaces.

The physical stability of the ELF-97 phosphate precipitate for extracellular labeling applications was analyzed in Table 1. ELF-97 alcohol-labeled cells were subjected to increasing numbers of centrifuge washes and vigorous mixing. The signal-to-noise ratio of the ELF-97 alcohol was actually found to increase somewhat following a single

centrifuge wash and remained at the same level following multiple wash steps. Vortex mixing resulted in only a partial loss of signal, indicating that the precipitate is sufficiently stable at the reaction site for effective fluorescent immunolabeling.

The ELF-97 phosphate was also compatible with multiple immunolabeling of cell surface markers. In Figure 6, mouse thymocytes were labeled with CFL-anti-CD8, PE-anti-Fas, PE-Cy5-anti-CD4, and biotin-anti-Thy1.2, followed by AP-conjugated streptavidin and ELF-97 phosphate. The cells were then analyzed using an argon-ion laser at 488 nm for scatter measurement, CFL, PE, and PE-Cy5 dye

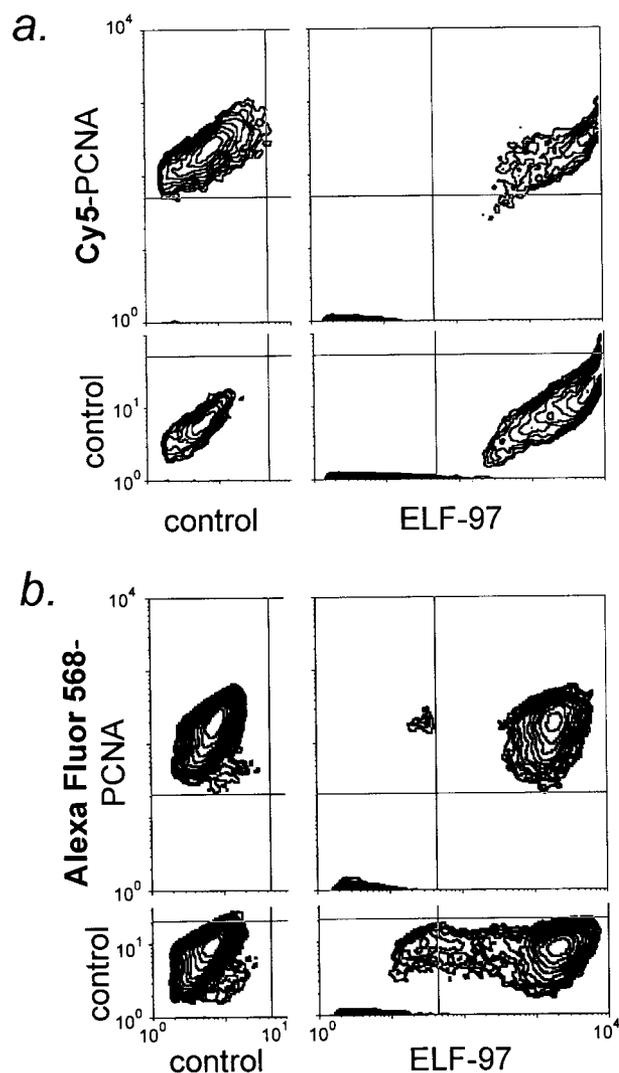


Fig. 3. Simultaneous analysis of Alexa Fluor 568 dye or Cy5 dye and ELF-97 alcohol fluorescence by flow cytometry. UMR-106 cells immunolabeled with unconjugated antibodies against PCNA followed by either a Cy5 dye- (a) or Alexa Fluor 568 dye (b) anti-mouse IgG secondary. The cells were subsequently labeled with ELF-97 phosphate to detect endogenous AP activity and analyzed by dual-beam flow cytometry as described in the Materials and Methods. Unlabeled and single-color controls are also shown. ELF-97 alcohol, Alexa Fluor 568 dye, and Cy5 dye fluorescence was analyzed on log scales.

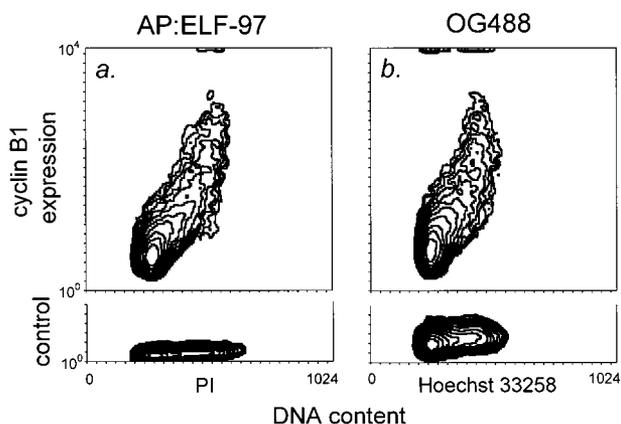


Fig. 4. Detection of intracellular cyclin B1 expression using ELF-97 phosphate and AP-conjugated labeling reagents. **a:** EL-4 mouse thymoma cells were labeled with anti-cyclin B1 followed by AP-conjugated anti-mouse IgG and subsequently labeled with the ELF-97 phosphate substrate (AP:ELF-97). Cells were then resuspended, stained with PI, and analyzed by dual-label flow cytometry as described in the Materials and Methods. **b:** For comparison purposes, EL-4 cells were labeled with anti-cyclin B1 as above followed by Oregon Green 488 dye anti-mouse IgG (OG488) as a secondary label and Hoechst 33258 nucleic acid stain for measurement of DNA content. Both labeling systems show elevated cyclin B1 expression in the G<sub>2</sub>/M cell cycle phase. ELF-97 alcohol, Oregon Green 488 dye, PI, and Hoechst 33268 fluorescence was analyzed on linear scales.

excitation and a krypton-ion laser emitting at 351 nm for ELF-97 alcohol excitation. As with the endogenous AP detection illustrated above, ELF-97 phosphate was spectrally compatible with fluorescein-based fluorochromes; it was also compatible with the phycobiliprotein PE and the PE-Cy5 dye tandem conjugate. Treatment with the ELF-97 buffer components did not appreciably alter the fluorescence intensity of either low molecular weight fluorophores (such as CFL) or phycobiliproteins (such as PE; data not shown). As above, control samples in which the AP-streptavidin was omitted showed no ELF-97 alcohol signal. These data therefore indicate that ELF-97 phosphate can be used as a fluorochrome in complex multi-color surface labeling applications with good spectral compatibility.

**DISCUSSION**

The ELF-97 phosphatase assay has proven to be superior to existing methods for detecting endogenous AP activity by flow cytometry (14,15). In the present studies, ELF-97 phosphate was successfully used to detect endogenous AP activity with simultaneous intracellular protein and DNA content measurement, and both intracellular and extracellular antigens using AP-conjugated-systems. The previously observed sensitivity of ELF-97 for endogenous AP was retained with simultaneous cell cycle protein and DNA content analysis, allowing the identification of a low AP subpopulation in the G1 cell cycle compartment of UMR-106 cells. This multiparametric assay accurately delineated the distribution of AP in cycling cells and was able to identify cell subsets with varying endogenous AP levels. ELF-97 phosphate can therefore be used to identify

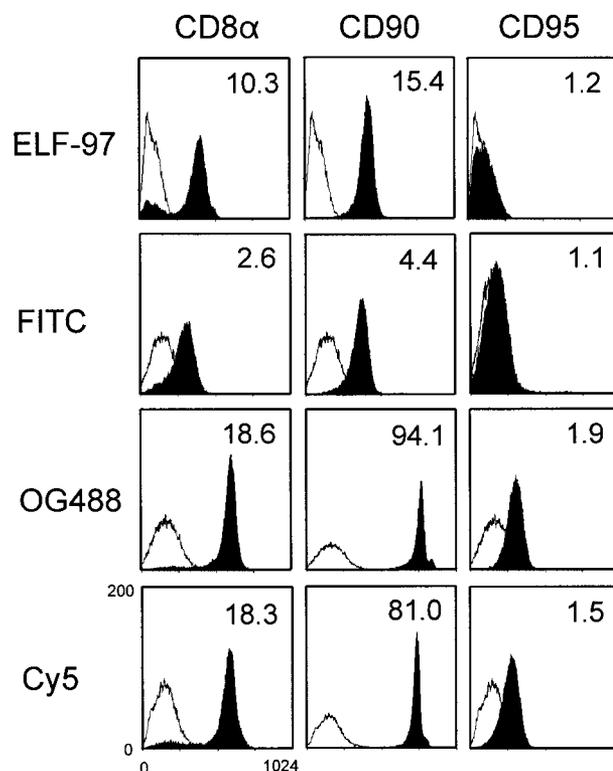


Fig. 5. Detection of cell surface marker expression using ELF-97 phosphate and AP-conjugated labeling reagents. Mouse thymocytes (unfixed) were labeled with biotin-conjugated antibodies against CD8 (left column), CD90/Thy1.2 (middle column), and CD95/Fas (right column), followed by secondary labeling with streptavidin conjugated with AP, FITC, Oregon Green 488 dye (OG488), and Cy5 dye. The AP-streptavidin-labeled cells were then labeled with the ELF-97 phosphate substrate. All cells were analyzed by dual-laser flow cytometry as described in the Materials and Methods. The resulting histograms show total fluorescent signal (filled peaks) versus isotype-matched control antibody samples (unfilled peaks). MFI ratios (total fluorescence divided by control) are shown in the upper right corner of each histogram. All fluorochromes were analyzed on log scales.

Table 1  
*Extracellular Labeling With ELF-97 Phosphate Following Multiple Cell Washing and Agitation\**

Sample treatment	MFI ratio
No wash	16
1× sample wash	18
2× sample wash	16
3× sample wash	15
3× sample wash and vigorous vortex	10

\*Mouse thymocytes (unfixed) were labeled with biotinylated anti-mouse Thy1.2 followed by AP-conjugated streptavidin and ELF-97 phosphate labeling. Cells were then either left unwashed (no wash), washed by centrifugation up to three times (1× wash, 2× wash, or 3× wash), or washed three times followed by vigorous vortex mixing (3× wash and vigorous vortex). They were analyzed by dual-laser flow cytometry as described in the Materials and Methods. Data are expressed as MFI ratios (total fluorescence divided by control) at right.

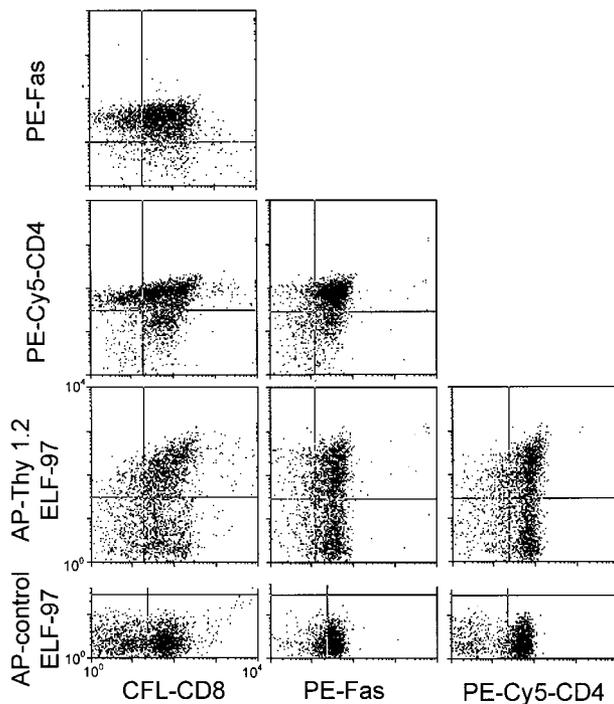


Fig. 6. Multicolor extracellular labeling with ELF-97 phosphate. Mouse splenocytes were labeled with CFL-anti-mouse CD8, PE-anti-mouse Fas, PE-Cy5 dye-anti-mouse CD4, and biotin-anti-mouse Thy1.2, followed by AP-conjugated streptavidin. The cells were then labeled with ELF-97 phosphate and analyzed by dual-laser flow cytometry for all four fluorochromes. All two-parameter cytograms are shown, as well as one-color controls for CFL, PE, and PE-Cy5 dye versus ELF-97 phosphate fluorescence. All fluorochromes were analyzed on log scales.

cell subsets with varying levels of AP activity in the context of cell cycle analysis. AP expression has been observed to vary with the level of differentiation in osteoblasts. *In vivo* AP-positive cells include differentiated osteoblasts, preosteoblasts, and osteoprogenitor cells at different stages of differentiation. Combining AP analysis with DNA content and cell cycle-associated protein expression may provide a basis for separating osteoblasts and their progenitors at different levels of differentiation. Labeling was found to be physically stable in both cases; this was particularly important in extracellular labeling, where the ELF-97 alcohol precipitate must remain associated with the membrane reaction site to give adequate detection.

ELF-97 phosphate was used successfully to detect intracellular antigen expression in ethanol-treated cells using unconjugated primary antibodies and AP-conjugated secondary reagents. By combining detection of a cell cycle-regulated intracellular antigen with simultaneous DNA content analysis, we found that detection by ELF-97 phosphate showed the same cell cycle specificity as that detected by direct immunolabeling. High endogenous AP levels (such as those found in UMR-106 cells) would obviously interfere with the detection of AP-conjugated secondary reagents. As was found to be the case for histochemical labeling (11), detection of AP labeling in the

presence of endogenous AP should be possible with levamisole or heat treatment, which would presumably block endogenous AP activity but not conjugated AP (usually derived from intestinal sources).

ELF-97 phosphate was also successfully used as an extracellular label, both alone and in combination with other fluorochromes. Because extracellular immunophenotyping of lymphocytes required cells to be in a viable state (in contrast to the intracellular labeling described above), the ELF-97 phosphatase labeling system needed to be sufficiently gentle to cause minimal cell damage (and subsequent alterations in surface marker expression). We found ELF-97 phosphate to be compatible with surface immunophenotyping, having little or no effect on previously attached antibodies and fluorochromes. Physical stability of the ELF-97 alcohol precipitate was a concern in these studies. Although ELF-97 phosphate has been previously used for extracellular labeling in imaging applications, the shear forces associated with sample preparation and actual flow analysis are considerably greater than in microscopy. The surface deposition of the ELF-97 alcohol precipitate was found to be quite stable during sample preparation, withstanding repeated centrifugation and washing. The effects of the shear forces associated with actual flow cytometric analysis were not evaluated but should be kept in mind as another source of physical stress on the precipitate association with cells. As with the intracellular labeling experiments, the endogenous AP activity present in the labeled cells should be evaluated beforehand and needs to be minimal for simple labeling. Again, levamisole may be used to block any endogenous AP activity present. Additionally, the ELF-97 phosphate reaction buffer has a high concentration of salt. It is unknown whether the brief incubation period required for the substrate reaction affected cell viability. Using the ELF-97 phosphate system for viable cell sorting (as opposed to analysis) should therefore be approached with caution. Because the ELF-97 phosphate system is extremely sensitive for the detection of endogenous AP, alterations in the labeling conditions might improve sensitivity for extracellular labeling, as well as improve cell viability. These experiments are currently in progress.

Spectral compatibility of ELF-97 phosphate with other fluorescent reagents is an essential factor in gauging its usefulness as a flow cytometric fluorochrome. Importantly, the ELF-97 phosphate was spectrally compatible with a variety of fluorochromes. These included the DNA binding dyes PI, 7-AAD, TO-PRO-3, BO-PRO-3, and the phenotyping fluorochromes fluorescein, Oregon Green 488 dye, PE, the PE-Cy5 dye tandem conjugate, the 568-nm excited Alexa Fluor 568 dye, and the 632-nm excited Cy5 dye. This result is consistent with previous histological fluorescence and confocal microscopy studies, which have shown ELF-97 phosphate to be spectrally compatible with fluorescein, tetramethylrhodamine, PI, the Hoechst dyes, and others (8,9,11). All of the above studies indicate that ELF-97 phosphate is a viable option for fluorescent immunophenotyping, expanding the utility of UV excitation in multilaser flow cytometry.

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