

# Pairs of Violet-Light-Excited Fluorochromes for Flow Cytometric Analysis

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We describe pairs of fluorochromes for use with the 407-nm line of a violet-light-enhanced krypton ion laser. These fluorochromes and a previously described violet-light-excited reporter variant, GFP-Vex, fall into two emission classes: blue for Cascade Blue, and green/yellow for Cascade Yellow, Lucifer Yellow, and GFP-Vex. Cascade Yellow is a new fluorochrome that we have synthesized and is used for the first time in the present study. The two emission classes are sufficiently different that Cascade Blue can be paired with Cascade Yellow, Lucifer Yellow, or GFP-Vex in flow cytometric analysis. Furthermore, with proper detection filters, these fluorochromes can be combined with all of the currently

used fluorochromes in a three-laser FACS system. With these data, the total number of fluorochromes that can be used as antibody labels for simultaneous detection in combined FACS analysis increases to nine. This study demonstrates the sensitivity and power of the combined use of these reagents in a single eight-color analysis by identifying murine T-lymphocyte subsets that could not otherwise be readily distinguished. *Cytometry* 33:435–444, 1998. © 1998 Wiley-Liss, Inc.

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A large number of phenotypic and functional markers exists to identify subpopulations of lymphocytes *ex vivo*. Because of the limitations in the number of fluorochromes available, however, it often is not possible to measure all desired parameters simultaneously. For FACS analysis, a suitable fluorochrome is bright, can be conjugated to proteins, can be excited by an available laser line, and is spectrally distinguishable from other fluorochromes. Only fluorochromes that are excited in the blue (488-nm argon ion line) and red ( $\approx$ 600-nm dye laser line or 632-nm He-Ne line) have met these criteria. Ultraviolet-light-excited fluorochromes have to contend with the higher autofluorescence of cells, making sensitive detection for immunophenotyping difficult, and far-red-light-excited fluorochromes cause problems with detector efficiency.

The violet-enhanced krypton laser, with lines at 407 nm and 413 nm, makes it possible to incorporate violet-light-excited fluorochromes for immunophenotyping. These lines lie midway between nucleotide and flavin excitation peaks, so that only low levels of autofluorescence are elicited from these lines (1–3). In addition, as shown in the present study and in the study by Roederer et al. (4), they are sufficiently isolated in wave length from the nearest commonly used 488-nm argon line to produce only low levels of spectral overlap between the signals from the

violet-light- and blue-light-excited fluorochromes. Moreover, with proper detection filters, the commonly used blue-light- and red-light-excited fluorochromes result in minimally detectable emission from the violet krypton lines.

The use of violet excitation on the flow cytometer has been demonstrated with GFP reporters (5,6) and antibody conjugates (4). Expression of the GFP variant GFP-Vex has been detected from as little as a single copy of its gene. In these studies, the krypton laser (407 nm) was configured on a flow cytometer with an argon laser (488 nm) and a dye laser (595 nm). We present data on three violet-light-excited fluorochromes—Cascade Blue, Cascade Yellow, and Lucifer Yellow—and demonstrate their suitability for combined analysis with blue-light- and red-light-excited fluorochromes.

Cascade Blue, the brightest of these fluorochromes, emits in the blue spectrum. It has a high extinction

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coefficient ( $27,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and a high quantum yield (0.5), making it approximately one-half as bright as fluorescein (7). This fluorochrome is not yet widely used in flow cytometry despite its wide use in immunofluorescence microscopy, presumably due to the lack of an appropriate laser line. In previous work Cascade Blue was excited in the ultraviolet (UV) spectrum, where it has a lower extinction coefficient than in the violet spectrum. Consequently, in those previous studies, it was found to be a dull stain (8). As we report in detail in the present study, excitation of Cascade Blue in the violet spectrum increases its brightness dramatically. We have previously shown (4) that Cascade Blue can be combined in FACS analysis with blue-light- and red-light-excited fluorochromes. In the present report, we describe the fluorochrome substitution levels that make Cascade Blue antibody conjugates optimally bright and demonstrate the use of these conjugates for FACS analysis in combination with other violet-light-excited fluorochromes.

The second of these fluorochromes, Cascade Yellow, a green/yellow emitter, is also relatively bright. It has a high extinction coefficient ( $25,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and a quantum yield of 0.56 (Haughton et al., unpublished observations). In studies on its parent, PyMPO (9), we obtained only low yields of PyMPO antibody conjugates from the gel filtration columns used for their purification (Anderson, unpublished observations). Furthermore, PyMPO conjugates had a tendency to self-aggregate. These difficulties were largely surmounted by the synthesis of the more hydrophilic Cascade Yellow, which was created by sulfonating the methoxyphenol group of 1-(3 (succinimidyl)oxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium bromide (PyMPO, succinimidyl ester (SE)).

The third violet-light-excited fluorochrome, Lucifer Yellow, is also a green/yellow emitter (10). It is not anticipated to be as bright as either Cascade Blue or Cascade Yellow because it has a lower extinction coefficient ( $12,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and lower quantum yield (0.25). Although its use in FACS analysis has not been described, it is used widely for immunofluorescence microscopy.

In the present study, we describe the use of pairs of violet-light-excited fluorochromes for FACS analysis, thus raising to nine the total number of fluorochromes that can be used as antibody labels for detection in combined FACS analysis.

## MATERIALS AND METHODS

### Reagents

Cascade Blue acetyl azide, Cascade Yellow succinimidyl ester, Cascade Blue-streptavidin, and Lucifer Yellow-avidin were obtained from Molecular Probes, Inc. (Eugene, OR). Streptavidin was purchased from Vector Laboratories Inc. (Burlingame, CA). Anhydrous dimethyl sulfoxide (DMSO) was obtained from Aldrich (Milwaukee, WI) and stored desiccated at room temperature. The antibacterial, antifungal growth inhibitor, pentachlorophenol (pHIX), was purchased from Pierce (Rockford, IL). Deficient RPMI medium lacking phenol red, biotin, and flavins was obtained from Irvine Scientific (Santa Ana, CA).

### Antibody Conjugation

In preparation for the conjugation of Cascade Blue and Cascade Yellow to the 53.6.7. monoclonal antibody (mAb; anti-mouse CD8 $\alpha$ ) (11), the antibody was dialyzed against 100 mM sodium carbonate, pH 8.2, and concentrated to 4 mg/ml. Immediately before the conjugation reaction, the fluorochrome reagent, Cascade Blue acetyl ester or Cascade Yellow succinimidyl ester, was resuspended in DMSO to a final concentration of 10 mg/ml. Conjugation reactions were begun by the dropwise addition of 10–250  $\mu\text{g}$  of fluorochrome to 1 mg of antibody, maintained at 4°C, and protected from light. After addition of fluorochrome, the tube containing the reaction mixture was placed on a slow rotator and kept at room temperature. At the end of the 4-h reaction, the process was stopped by separation of the conjugate product from the unbound fluorochrome on a PD 10 gel filtration column (Pharmacia, Piscataway, NJ), preequilibrated with 10 mM Tris, 150 mM NaCl, and pHIX. Column fractions containing the conjugate were identified by measuring their absorbance at 280 nm and then pooled. The conjugation of Cascade Yellow to streptavidin was done in a similar manner: 1 mg of streptavidin was dissolved in 0.5 ml of 100 mM sodium carbonate, pH 8.2. Conjugation reactions were begun by the addition of 12.5–100  $\mu\text{g}$  of fluorochrome to 0.25 mg of streptavidin. The brightest reagents were obtained under the following conditions: Cascade Yellow-streptavidin, addition of 25  $\mu\text{g}$  Cascade Yellow succinimidyl ester; Cascade Yellow-antibody conjugates, addition of 40  $\mu\text{g}$  of Cascade Yellow succinimidyl ester; Cascade Blue-antibody conjugates, addition of 60  $\mu\text{g}$  of Cascade Blue acetyl ester. The other conjugates used in this study were either obtained from Pharmingen (San Diego, CA) or Molecular Probes, Inc. or were prepared by previously described methods (12,13). Cascade Blue and Cascade Yellow conjugates are used routinely in our laboratory and have been proven stable for more than one year.

### Fluorochrome/Antibody Substitution Levels, Signal-to-Background Evaluations, and Quantum Yields

The fluorochrome/antibody substitution levels (f/p) were defined as the molar amount of fluorochrome divided by the molar amount of antibody. Fluorochrome concentrations were calculated by dividing absorbance, measured at 400 nm for Cascade Blue and at 410 nm for Cascade Yellow and Lucifer Yellow, and by the extinction coefficient:  $27,000 \text{ cm}^{-1} \text{ M}^{-1}$ ,  $25,000 \text{ cm}^{-1} \text{ M}^{-1}$ , and  $12,000 \text{ cm}^{-1} \text{ M}^{-1}$ , respectively. Protein concentrations were determined with the Bio-Rad DC colorimetric assay (Bio-Rad Laboratories, Hercules, CA). The fluorochromes did not interfere with the measurement of the protein assay because they do not absorb significantly at 595 nm. The quantum yield of the free Cascade Yellow dye was determined by using quinine sulfate in 0.5 M sulfuric acid as the reference standard (quantum yield = 0.56).

A previously described statistical analysis was performed to determine signal-to-background (sig/bkd) values

for the different conjugates analyzed in the present study (14).

### Preparation of Murine Primary Cells and Cell Cultures

Single-cell suspensions of splenocytes were obtained from BALB/c mice by standard procedures. Lung parenchyma cells from normal mice and mice infected with the influenza virus A/Mem71 were obtained by collagenase/DNAse digestion, as described previously (15). For staining, cells were resuspended at a final concentration of  $2.5 \times 10^7$  cells/ml deficient RPMI staining media supplemented with 3% newborn calf serum, 10 mM HEPES, pH 7.2, 0.2% sodium azide, and 1 mM EDTA. The Abelson Murine Leukemia Virus-transformed pre-B cell line 1-8 (16) was cultured in RPMI medium (Gibco BRL, Grand Island, NY) supplemented with 100 units/ml penicillin, 50 nM streptomycin (Gibco BRL), 2 mM glutamine, 50 nM  $\beta$ -mercaptoethanol, and 10% fetal calf serum (Hyclone Laboratories Inc. Logan, UT) at 37°C in 5% CO<sub>2</sub>. Cell line 1-8 was infected with the MFG-GFP-Vex2 murine retrovirus as described elsewhere (6) except that, after the addition of virus-containing supernatant to the cells, the cells were centrifuged at 1,000*g* at room temperature for 60 min (6,17,18).

### Cell Staining

Cells were directly stained with antibody conjugates by incubating the cells with previously determined saturating amounts of antibodies on ice for 20 min, followed by two washes in staining medium. Indirect staining reactions with biotinylated antibodies and fluorochrome-labeled avidin and streptavidin conjugates were performed identically, except that the avidin and streptavidin conjugates were added after the first set of washes, incubated on ice for 15 min, and then washed. After the final wash, cells were resuspended in staining medium containing 1  $\mu$ g/ml propidium iodide (PI).

### Flow Cytometric Analysis

The hybrid three-laser flow cytometer used for the present studies is detailed elsewhere (4). Briefly, this flow cytometer is composed of a modified FACStar Plus (Becton Dickinson, San Jose, CA) attached to a standard MoFlo electronics console (Cytomation, Inc., Fort Collins, CO) and custom electronics developed by the FACS Development Group at Stanford. It is configured with three lasers: an Innova 300 violet-light-enhanced krypton laser (Coherent, Inc., Palo Alto, CA) tuned to 407 nm at 180 mW of power for violet excitation; an Innova I-90 (Coherent, Inc.) argon ion laser run in all-lines mode at approximately 5 W; and a dichroic filter (CVI Laser Corp., Livermore, CA) is used to split off about 250 mW of 488-nm light to provide blue excitation at 488 nm and to pump a dye laser (Coherent, Inc.) to produce approximately 500 mW at 595 nm. The emission detection filters for the 488-nm excited dyes and the 595-nm excited dyes are used as described in a previous report (4). The violet emissions are split with a 490-nm dichroic filter; the Cascade Blue emission is

collected with a 440/40-nm bandpass filter, the GFP-Vex2 emission is collected with a 515/40-nm bandpass filter, and the Cascade Yellow and Lucifer Yellow emissions are collected with a 535/40-nm bandpass filter (all emission filters are from Omega Optical, Brattleboro, VT). Multiparameter data were collected by using FACS-Desk software (19) and analyzed by using either FACS/Desk or Flow Jo software. Flow Jo was developed by Adam Triester, Wayne Moore, David Parks, Marty Bigos, and Mario Roederer (Stanford FACS Development Group) and is available through Treestar, Inc. (San Carlos, CA).

## RESULTS

### Violet-Light-Excited Fluorochromes Are Best Used With the 407-nm Line of the Krypton Laser

The Innova 300 krypton laser has two violet lines, a 407-nm line and a 413-nm line that can be used either individually or in combination. To identify the best operating condition for use with the violet-light-excited fluorochromes, we first examined the spectra of these fluorochromes (Fig. 1). This examination showed that both the 407-nm and the 413-nm line were located within the excitation peaks of Cascade Yellow, Lucifer Yellow, and GFP-Vex, whereas only the 407-nm line was located within the narrow excitation peak of Cascade Blue.

Splenocytes indirectly stained with biotinylated anti-murine CD8 and the streptavidin conjugates of Cascade Blue and Cascade Yellow or the avidin conjugates of Lucifer Yellow confirmed the superiority of the 407-nm line for excitation of these violet-light-excited fluorochromes (Fig. 2). The conjugates were evaluated for brightness with the sig/bkd method described by Parks and Bigos (14). As anticipated, the Cascade Blue conjugate produced much greater levels of fluorescence signals (2.5-fold greater sig/bkd values) when excited at 407 nm than when excited at 413 nm. In contrast, staining with Cascade Yellow and Lucifer Yellow produced similar levels of fluorescence signal when excited at either 407 or 413 nm. Consequently, the 407-nm line was used in all further studies. The emissions of the violet-light-excited fluorochromes, Cascade Yellow and Lucifer Yellow, were collected with the 535/40-nm bandpass filter. Use of this filter minimized the interlaser overlaps from the blue-light-excited fluorochromes into the Cascade Yellow-Lucifer Yellow detector (Fig. 4).

### Optimization of Cascade Blue and Cascade Yellow Conjugates

To optimize Cascade Blue and Cascade Yellow antibody conjugates, we made a series of anti-CD8 reagents that differed in the level of fluorochrome substitution. The brightness of each conjugate was determined by calculating the sig/bkd value of stained murine splenocytes run on FACS (Fig. 3). Each Cascade Blue and Cascade Yellow conjugate resolved the expected number of cells from the pool of lymphocytes (7.2–10% of gated cells). The staining was specific and quantitative, as determined by comparison to an anti-mouse CD8-fluorescein isothiocyanate (FITC)

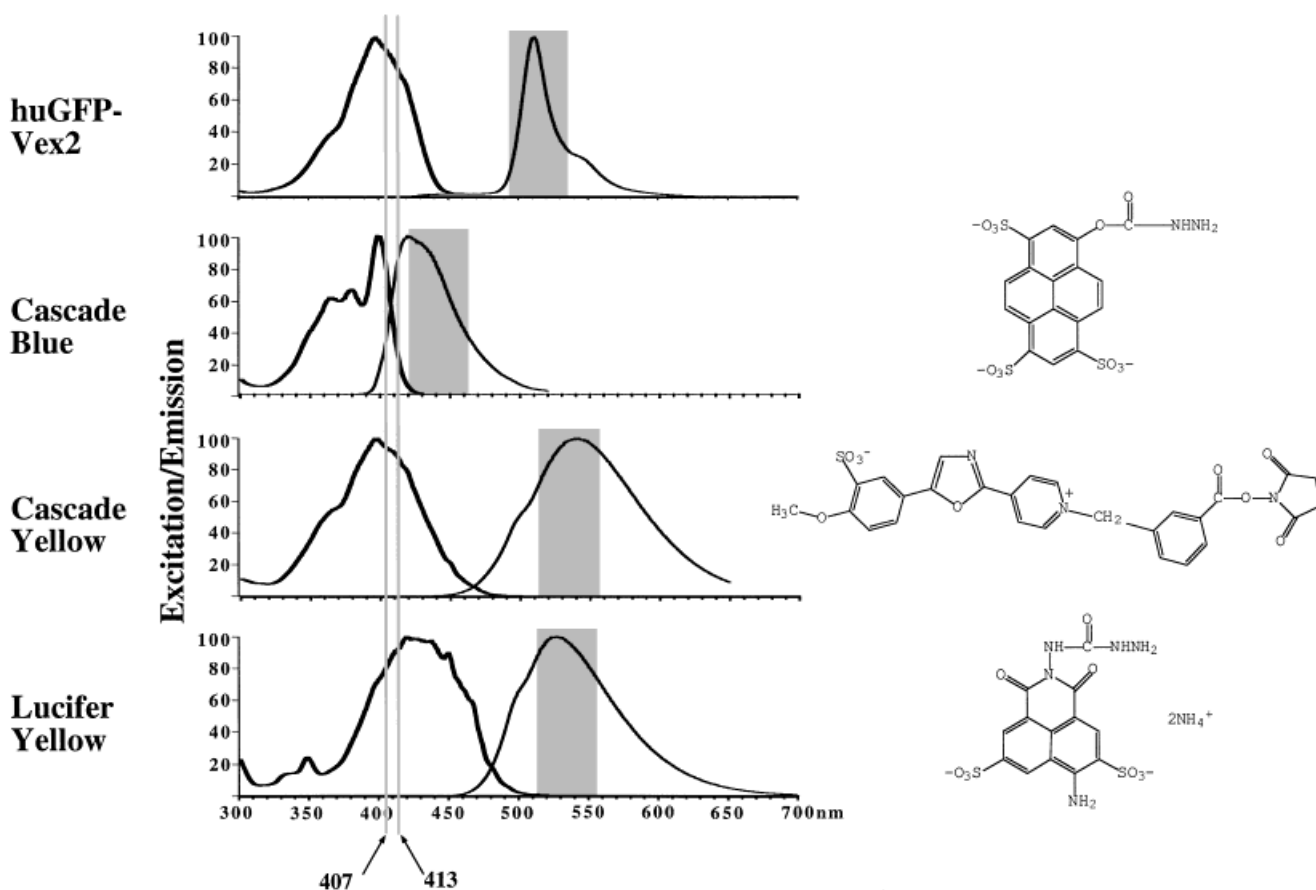


FIG. 1. Absorbance and fluorescence spectra of the anti-CD8 antibody conjugates of Cascade Blue, Lucifer Yellow, and Cascade Yellow. The gray boxes indicate the wave lengths of light passing through the following bandpass filters: 515/40 nm (GFP-Vex2), 440/40 nm (Cascade Blue), and 535/40 nm (Cascade Yellow and Lucifer Yellow).

conjugate that stained a similar frequency of cells (data not shown). The brightest member of each series had approximately five fluorochrome substitutions per antibody and produced fluorescent signals comparable to those of fluorescein; sig/bkd values obtained with the Cascade Blue anti-CD8 conjugate were 80% and those obtained with the Cascade Yellow anti-CD8 conjugate were 40% of those obtained with an optimized fluorescein anti-CD8 conjugate (data not shown). Sub- or supraoptimal levels of fluorochrome substitution resulted in duller staining; in particular Cascade Blue conjugates were maximally bright over a relatively narrow range of substitution ratios.

Lucifer Yellow also proved useful for FACS analysis when tested as indirect stain. This was shown with an Lucifer Yellow-avidin conjugate in conjunction with a biotinylated anti-murine CD8 antibody (Fig. 2). FACS analysis of splenocytes showed that CD8 T cells were completely resolved from the lymphocyte pool. As anticipated, staining with Lucifer Yellow-avidin was relatively dull, with sig/bkd values 31% and 46% of those seen with Cascade Blue-streptavidin and Cascade Yellow-avidin (Fig. 2). The fluorochrome substitutions for these optimized conjugates were as follows: 2 molecules Lucifer

yellow per avidin, 3.5 molecules Cascade Blue per streptavidin, and 5.4 molecules Cascade Yellow per streptavidin.

#### Spectral Overlaps in Multiparameter Analysis

To examine the suitability of these violet-light-excited fluorochromes for use in multiparameter analysis, we examined the intralaser overlaps between the violet-light-excited fluorochromes and the interlaser overlaps between the violet-light- and the blue-light-excited fluorochromes. These overlaps were identified by separately staining murine splenocytes with the conjugates of violet-light- and blue-light-excited fluorochromes and collecting their fluorescent signals at five separate detectors. Representative histograms are depicted in Figure 4.

The data showed that Cascade Blue can be paired with either Cascade Yellow or Lucifer Yellow because of the minimal emission overlaps that occur between these pairs (Fig. 4). Furthermore, neither Cascade Blue nor Cascade Yellow produced signals above background levels at the fluorescein and PE detectors. The overlap of the violet-light-excited fluorochrome Lucifer Yellow into the fluorescein and PE detectors is more significant because Lucifer Yellow is excited by the 488-nm line to emit 25–50% of its

FIG. 2. Comparison of the 407-nm and 413-nm excited (Ex) signals obtained from Cascade Blue, Cascade Yellow, and Lucifer Yellow conjugates. Mouse splenocytes were indirectly stained with biotinylated anti-CD8 antibody and then with Cascade Blue-streptavidin, Cascade Yellow-streptavidin, and Lucifer Yellow-avidin. The 407-nm excited signals were collected with a 440/40-nm bandpass filter (for Cascade Blue) and a 535/40-nm bandpass filter (for Cascade Yellow and Lucifer Yellow). After application of FSC/SSC lymphocyte gates, signal-to-background values of the positively stained cells were calculated and are shown.

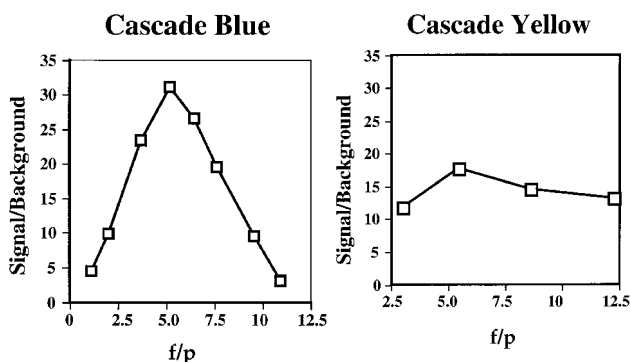
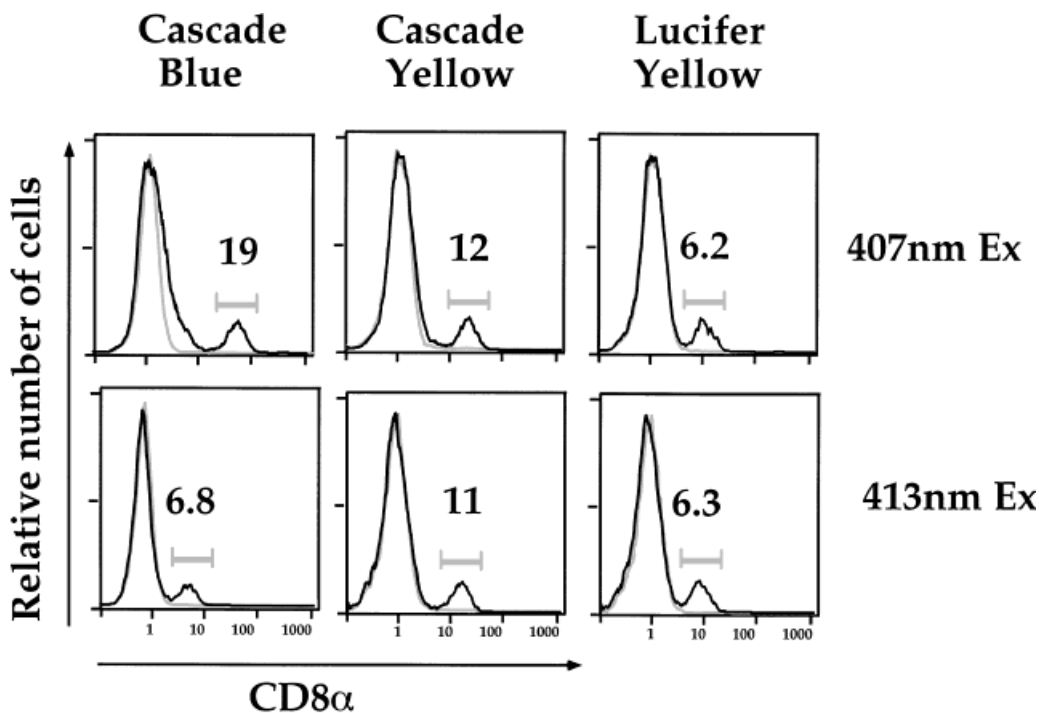


FIG. 3. Effects of fluorochrome substitution levels on Cascade Blue and Cascade Yellow conjugates. Shown are the signal-to-background (sig/bkd) values for anti-CD8 Cascade Blue and Cascade Yellow antibody conjugates produced by using different fluorochrome/protein ratios ( $f/p$ ). Sig/bkd values were derived as described previously (14).

maximally detected signal at these detectors. Conversely, the blue-light-excited fluorochromes produced only low-level signals at the violet-light-excited fluorochrome detectors.

#### Pairing of Cascade Blue With Lucifer Yellow

To test whether Cascade Blue and Lucifer Yellow could be paired for FACS analysis, we stained mouse splenocytes with a Cascade Blue-labeled anti-CD4 T-cell antibody (G.K.1.5) and a biotinylated anti-CD45R antibody (RB3-6B2, anti-B220 predominantly staining B cells), which was demonstrated with avidin-Lucifer Yellow. FACS analysis of the stained splenocytes (Fig. 5A) showed that the Cascade Blue and the Lucifer Yellow conjugates unambiguously

distinguished the CD4<sup>+</sup> T cells and the B lymphocytes, respectively.

#### Pairing of Cascade Blue With the GFP-Vex Reporter

To expand the repertoire of violet-light-excited fluorochrome pairs for FACS analysis, we tested whether antibodies labeled with Cascade Blue could be paired with GFP-Vex reporters. Transfection of the 1-8 pre-B cell line with MFG-huGFP-Vex2 retrovirus resulted in a heterogeneous cell population, in which 60% of the cells expressed the huGFP-Vex2 reporter protein (18). These cells were stained with biotinylated antibody AA4.1 that recognized an unknown surface marker found on stem cells (20), B-cell precursors (21,22), bi-potent progenitors (23,24), and streptavidin-Cascade Blue. Controls were stained with streptavidin-Cascade Blue only. FACS analysis showed that cells stained for AA4.1 and that control cells were clearly distinguishable, regardless of the coexpression levels of huVex-2 GFP (Fig. 5B).

#### Eight-Color Multiparameter Analysis With Cascade Blue and Cascade Yellow Conjugates

Figure 6 shows an example of an eight-color analysis that uses the two violet-light-excited fluorochromes Cascade Blue and Cascade Yellow with the well-established blue-light- and red-light-excited dyes FITC, PE, indocarbocyanine (Cy5-PE), TR, and APC and the tandem dye Cy7-APC. The aim of the experiment was to determine (a) whether differences in the frequencies of activated T cells exist between VB8.1/8.2<sup>+</sup> and VB8.1/8.2<sup>-</sup> T cells after infection with influenza virus because preliminary results had suggested that VB8.1/8.2<sup>+</sup> T cells preferentially increase during influenza virus infection (Baumgarth, unpub-

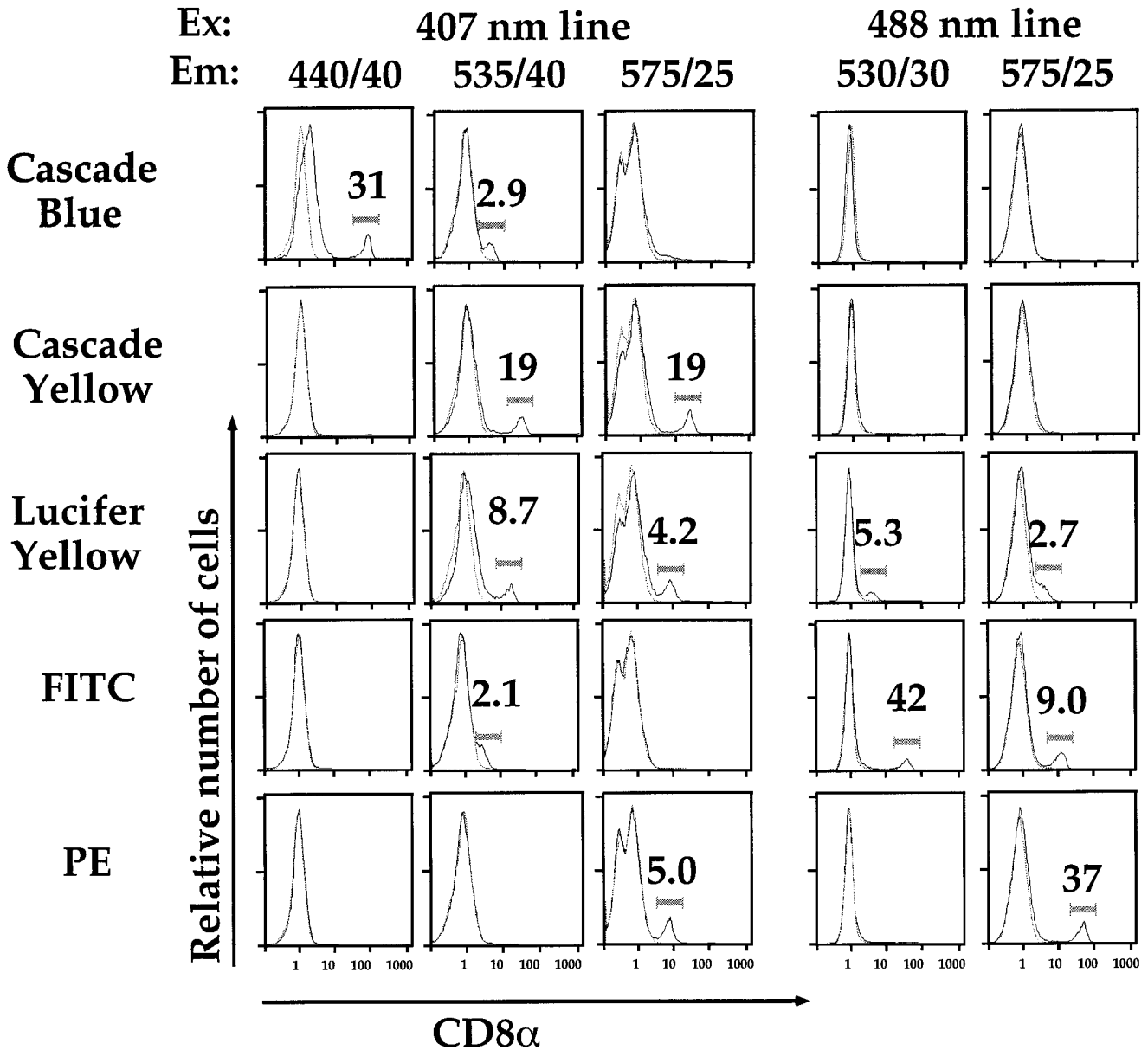


FIG. 4. Identification of spectral overlaps among the violet-light- and blue-light-excited fluorochromes. Murine splenocytes were stained either directly with anti-CD8 antibody conjugates of Cascade Blue, Cascade Yellow, fluorescein, or PE or indirectly with biotinylated anti-CD8 antibody and Lucifer Yellow-avidin. The 407-nm excited signals of these conjugates were collected with 440/40-nm, 535/40-nm, and 575/30-nm bandpass filters. The 488-nm excited signals were collected with 530/30-nm and 575/35-nm bandpass filters. Cells were gated for lymphocyte FSC/SSC and the signal-to-background values of the positively stained cells were determined and are shown.

lished observations) and (b) whether further phenotypic heterogeneity exists between activated T cells in lung and lung parenchyma because these cells are functionally remarkably different (15,25). After exclusion of P.I.-positive cells (Fig. 6A), Vb8.1/8.2<sup>+</sup> CD8<sup>+</sup> T cells were clearly identified from spleens and lungs of normal and day 7 influenza-virus-infected mice by gating on CD3<sup>+</sup>, IgM<sup>-</sup> CD45R<sup>-</sup> (B220<sup>-</sup>; Fig. 6B) CD4<sup>-</sup> CD8<sup>+</sup> (Fig. 6C) and Vb8.1/8.2<sup>+</sup> cells (Fig. 6D). Staining for CD3 was included as a bona fide T-cell marker to identify CD8<sup>+</sup> T cells from

other cells that may express this marker (i.e., dendritic cells). Inclusion of CD4 into the staining mix allowed us to exclude CD4 and CD8 double-positive cells and to analyze CD4<sup>+</sup> single-positive T cells simultaneously in the same sample. Inclusion of a "dump" channel (in this case, containing the B-cell markers B220 and IgM) greatly increases the quality of the data because not only B cells but also autofluorescent cells can clearly be separated from the cells of interest. The expression of CD62L was studied on Vb8.1/8.2<sup>+</sup> CD8<sup>+</sup> T cells from spleens and lung

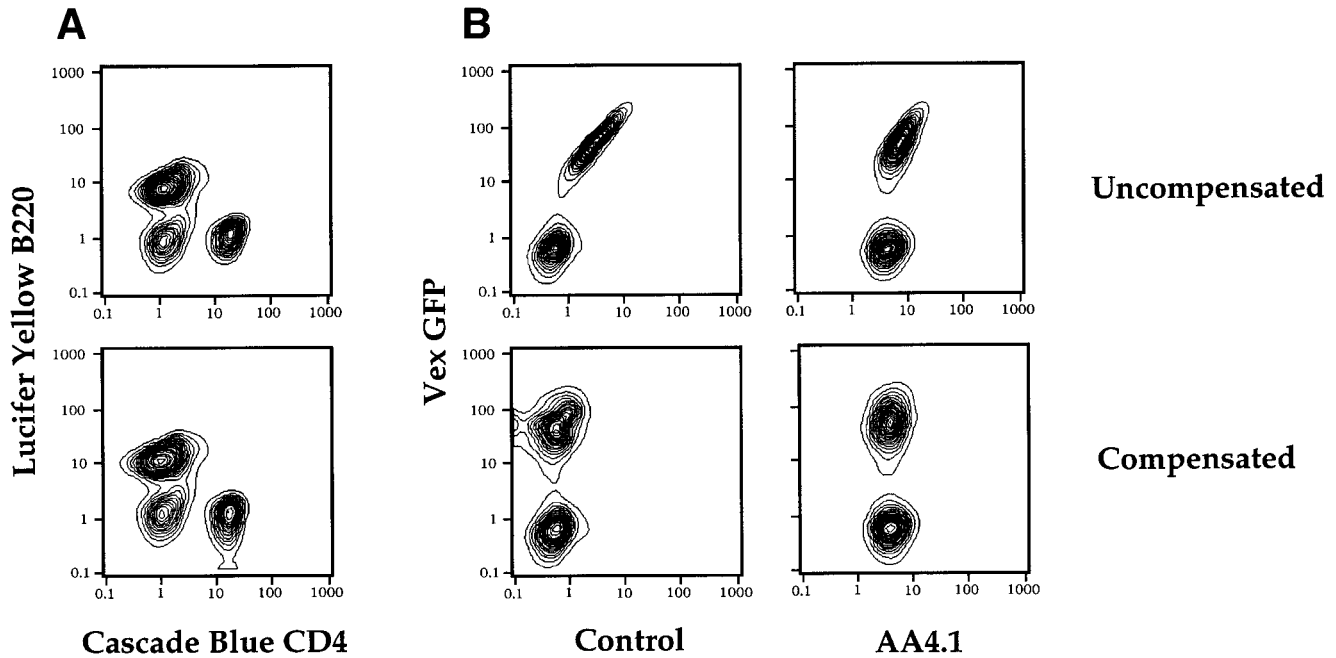


FIG. 5. Two-color FACS analysis using pairs of violet-light-excited fluorochromes. **A:** Shown are 5% probability contour plots of murine splenocytes stained with anti-CD4 Cascade Blue and biotinylated anti-B220 plus Lucifer Yellow-avidin. **B:** A pre-B cell line, heterogeneous in its expression of Vex-GFP and stained either with biotinylated AA4.1 antibody and Cascade Blue-streptavidin or with Cascade Blue-streptavidin only is shown after gating for lymphocyte FSC/SSC.

parenchyma of control mice and of mice 7 days after influenza virus infection and gated for high or low expression of the two activation markers CD44 and CD11a (Fig. 6E).

Influenza virus infection leads to a preferential increase in T cells, in particular CD8<sup>+</sup> T cells in the lung parenchyma of infected mice (26), but does not alter the frequencies of T cells in the spleen (data not shown). Phenotypically activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, identified as CD11a and CD44 double-high cells, have been reported to increase in the lung parenchyma, reaching highest levels at day 7 after infection (25). As determined in the present study, the relative frequencies of VB8.1/VB8.2<sup>+</sup> cells among CD8<sup>+</sup> T cells in control and influenza-infected mice were 29% and 30% in the spleen and 30% and 34% in the lung, respectively (data not shown). In previous studies, three-color analysis used to identify activation marker expression on T cells after infection with the influenza virus had suggested that CD62L surface expression was absent only on CD44 and CD11a double-high T cells (25). Simultaneous staining for all three activation markers confirmed this finding for splenic CD8<sup>+</sup> T cells. However, in the present study, a small population of CD8<sup>+</sup> lung T cells was identified which lacked CD62L expression and did not express high levels of CD44 and CD11a (Fig. 6). This population increased in size from 14% in controls to 24% after influenza virus infection. Similar increases in this activated T-cell population and in the CD44 and CD11a double-high T cell population were found among both VB8.1/8.2<sup>+</sup> and VB8.1/8.2<sup>-</sup>, CD8<sup>+</sup> T

cells (data not shown), suggesting that within the responding CD8<sup>+</sup> T cell pool VB8.1/8.2 TCR was not preferentially used.

## DISCUSSION

In the present report, we describe the use of pairs of violet-light-excited fluorochromes, including the fluorescent reporter gene GFP-Vex (6), for use in flow cytometry. These fluorochromes can be combined with the more commonly used blue-light- and red-light-excited dyes, raising to nine the total number of color markers available for multicolor FACS analysis.

Three fluorochromes, Cascade Blue, Cascade Yellow, and Lucifer Yellow, are each well excited by the 407-nm line of a violet-enhanced krypton laser. Cascade Blue, the brightest of these fluorochromes, emits in a narrow peak within the blue (7), and its sharp primary excitation peak limits its effective excitation to the 407-nm line. Previous studies using UV excitation for Cascade Blue found it to be a dull dye that required two-step staining to be useful at all for immunophenotyping (8). In contrast, we show that Cascade Blue excited by the 407-nm krypton line is a bright reagent that permits its use as a direct antibody label because it distinguishes autofluorescence and cell surface antigens expressed at even moderate densities. When the sig/bkd values for the Cascade Blue and fluorescein conjugates were calculated according to the method of Parks and Bigos (14), the results indicated that the Cascade Blue conjugate had 80% the brightness of the fluorescein conjugate. In addition, the narrow blue emission peak of Cascade Blue makes this fluorochrome amenable for use in

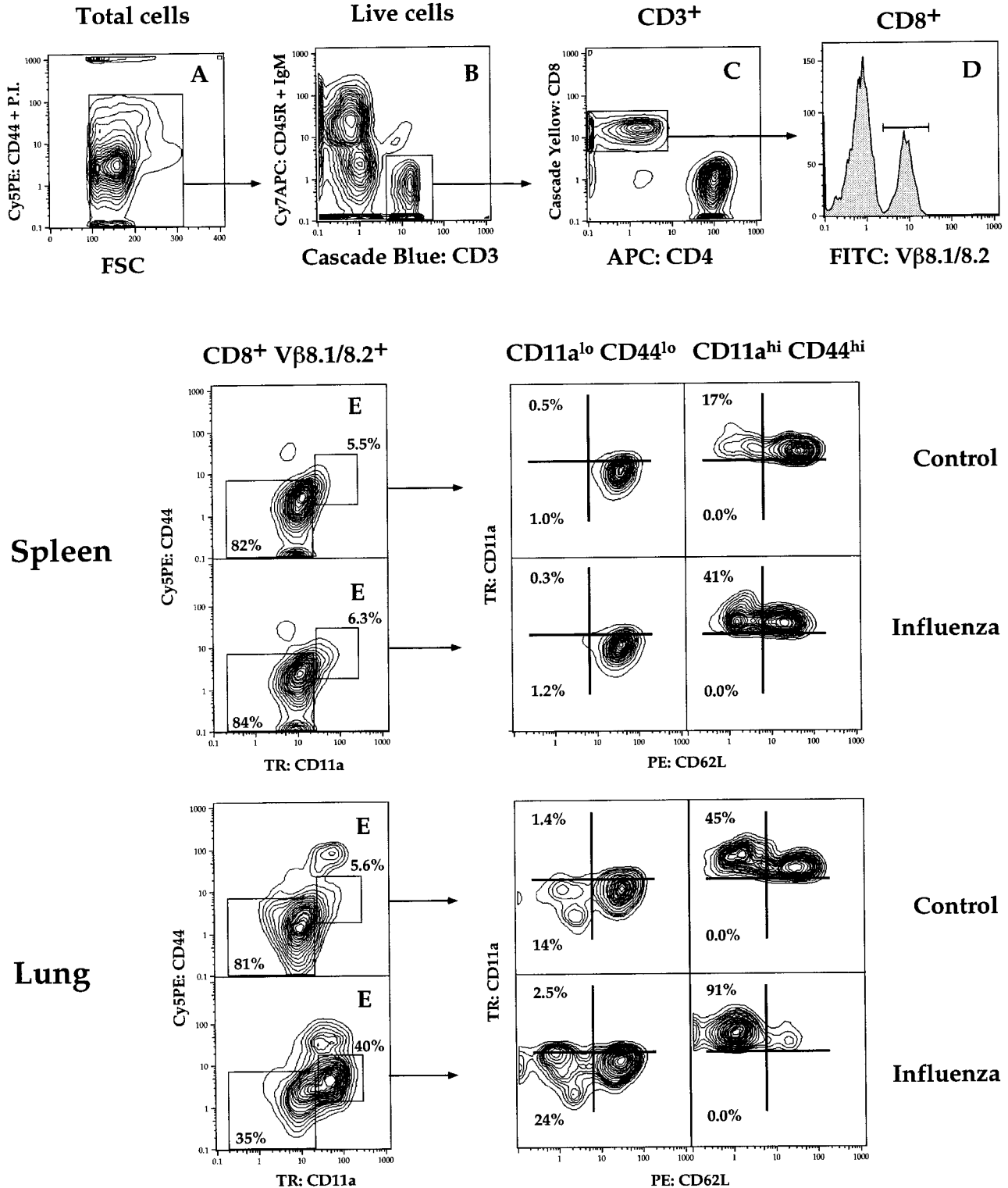


FIG. 6. Eight-color FACS analysis including Cascade Blue and Cascade Yellow reagents identified alterations in subpopulations of activated T cells from spleen and lung parenchyma after influenza infection. Single-cell suspensions from spleen and lung parenchyma of control mice and mice infected 7 days previously with influenza A/Mem71 virus were stained with antibodies to CD44 (biotinylated, plus Str.-Cy5-PE), CD45R and IgM (both Cy7-APC), CD3 (Cascade Blue), CD4 (APC), CD8 (Cascade Yellow), TCRVb 8.1/8.2 (fluorescein isothiocyanate), CD11a (LFA-1; PE), and CD62L (TR). The top panel shows the gates set (selected cells boxed) to identify live (P.I. negative) (A), CD3<sup>+</sup> and CD45R/IgM<sup>-</sup> (B), CD8<sup>+</sup> CD4<sup>-</sup>, (C) Vb8.1/8.2<sup>+</sup>, (D) T cells. Subpopulations of activated T cells were identified by the high surface expression of CD44 and CD11a (E). In contrast, resting T cells expressed low levels of CD44 and CD11a (E), as reported previously (25). Expression of CD62L is shown for both activated and resting Vb8.1/8.2<sup>+</sup> CD8<sup>+</sup> T cells.



multiparameter analysis in addition to the other violet-light-excited fluorochromes that emit in the green/yellow spectrum.

One of the examined green/yellow emitters, Cascade Yellow, is a newly synthesized fluorochrome. It is more hydrophilic and easier to conjugate to antibodies than its parent, PyMPO (Anderson, unpublished observations). Cascade Yellow emits in a broad peak in the green and yellow spectra. Its excitation peak is also broad, but is centered at a sufficiently short wave length to make it excitable by the 407-nm line but not by the 488-nm line. The sig/bkd calculations indicated that Cascade Yellow had approximately 40% the brightness of fluorescein, which is still of adequate brightness to detect cellular antigens expressed at moderate density. Another green/yellow emitter, Lucifer Yellow, is excited at slightly longer wavelengths than Cascade Yellow. Therefore, it is well excited by the 407-nm line but is also weakly excited by the 488-nm line in emitting fluorescence signals collected by fluorescein and PE detectors. Because these are minor signal overlaps, they should easily be compensated in most instances. The identification of two fluorochromes with widely different chemical structures and different physical characteristics but similar emission spectra should increase the flexibility for the synthesis of further derivatives of fluorochromes that can be combined with Cascade Blue.

A GFP variant, GFP-Vex, is a fourth fluorochrome excited by the violet lines. The reporter gene construct used in the present study was altered from the one originally described (6), thereby improving its signals approximately 10-fold (18). GFP-Vex is maximally excited at even shorter wavelengths than are the other fluorochromes, at approximately 395 nm. GFP-Vex emits in a relatively sharp peak at 509 nm and emits only low levels of fluorescence signals collected by the Cascade Blue detector, thus permitting it to be paired with Cascade Blue. The GFP proteins have found a wide range of uses including as *in vivo* reporter proteins in transgenic mice (4). The coupling of single-cell reporter gene detection and immunophenotyping, already described for the blue-light-excited GFP-Bex variant (4), should become a powerful tool in gene regulation studies.

While identifying optimal conditions for the conjugation of Cascade Blue and Cascade Yellow to antibodies, we found that the brightest Cascade Blue conjugates were obtained over a quite narrow range of fluorochrome substitution levels, whereas the brightness of Cascade Yellow conjugates was less affected by changes in the substitution levels (Fig. 3). This observation highlights the need for optimizing the levels of fluorochrome substitution for each conjugate to achieve maximally bright reagents. The brightest conjugates of both Cascade Blue and Cascade Yellow had an average of five fluorochrome substitutions on each antibody. At substitution levels below this, the conjugate was less fluorescent, presumably due to fewer fluorochromes per antibody molecule. Unlike fluorescein, for which self-quenching precludes substitution levels beyond about 2 or 3, other competing

influences lowered the signals of Cascade Blue and Cascade Yellow conjugates at substitution levels above 5 (7). One of the drawbacks of using Cascade Yellow for antibody labeling is that during conjugate preparation an unusual high amount of the reagent is lost, presumably because it sticks to the exclusion column. Although these losses were less than those observed with the parent compound, PyMPO succinimidyl ester (data not shown), greater amounts of antibody will be required to obtain adequate amounts of Cascade Yellow conjugates versus other dyes.

The number of parameters available for FACS analysis was limited by the number of fluorochromes that can be simultaneously excited and independently detected. We have shown that Cascade Blue and Cascade Yellow conjugates can be used simultaneously with FITC, PE, Cy5-PE, APC, and TR and the tandem dye Cy7-PE because the fluorescence emissions of Cascade Blue and Cascade Yellow can be detected almost completely independently of each other and independently of the 488-nm and 595-nm excited fluorochromes. The lack of interference of the violet-light-excited fluorochromes with those excited in the blue and the red extends by two the number of parameters available for simultaneous FACS analysis. Although the instrumentation is not yet available to detect more than eight colors simultaneously, ongoing efforts aim to increase this number. Substituting one or two blue-light- and red-light-excited fluorochromes by violet-light-excited dyes may also be advantageous because it should permit sensitive detection of cellular antigens by decreasing the signal overlaps between the fluorochromes. The reduction of interlaser signal overlaps is particularly important in analyses in which sorting decisions need to be made in real time.

The extraordinary sensitivity of eight-color FACS analysis was shown in our analysis of murine spleen and lung parenchyma cells. Three-color FACS analysis had suggested that expression of the homing marker CD62L was reduced only on those T cells that had acquired high level expression of CD11a and CD44 (25). Simultaneous staining of all three markers on heavily gated CD8<sup>+</sup> T cells has demonstrated a small population of lung cells that lacks expression of CD62L but shows low level expression of CD11a and CD44. Interestingly, this population increases after influenza virus infection, suggesting its involvement in the local immune response to the virus. The combination of surface phenotyping with intracytoplasmic cytokine staining can be used to unravel some of the potential *in vivo* functions of this CD62L<sup>-</sup>, CD11a, and CD44 double-low lung CD8<sup>+</sup> T cell population.

This study demonstrates the availability of various pairs of violet-light-excited fluorochromes for combined use with the commonly used blue-light- and red-light-excited dyes. Increasing the number of simultaneously usable dyes increases the power of multicolor FACS analysis. Collectively, these data highlight the importance of the 407-nm krypton laser line as an important new excitation for multicolor FACS.

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