

Analysis of Violet-Excited Fluorochromes by Flow Cytometry Using a Violet Laser Diode

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Received 9 October 2002; Revision Received 22 January 2003; Accepted 13 February 2003

Background: Low power violet laser diodes (VLDs) have been evaluated as potential replacements for water-cooled argon-ion and krypton-ion ultraviolet and violet lasers for DNA content analysis using the Hoechst dyes and 4,6-diamidino-2-phenylindole (Shapiro HMN, Perlmutter NG: *Cytometry* 44:133–136, 2001). In this study, we used a VLD to excite a variety of violet-excited fluorescent molecules important in biomedical analysis, including the fluorochromes Cascade Blue and Pacific Blue, the expressible fluorescent protein cyan fluorescent protein (CFP), and the fluorogenic alkaline phosphatase (AP) substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazoline (ELF-97; for endogenous AP detection and cell surface labeling with AP-conjugated antibodies).

Methods: Comparisons were made between VLD excitation and a krypton-ion laser emitting at 407 nm (both at higher power levels and with the beam attenuated at levels approximating the VLD) on the same FACSVantage SE stream-in-air flow cytometer. We evaluated a Power Technology 408-nm VLD (30 mW) equipped with circularization optics (18 mW maximum output, set to 15 mW)

and a Coherent I-302C krypton-ion laser emitting at power levels ranging from 15 to 75 mW.

Results: Cascade Blue, Pacific Blue, and CFP showed comparable signal-to-noise ratios and levels of sensitivity with VLD excitation versus the krypton-ion laser at high and VLD-matched power outputs. Multicolor fluorescent protein analysis with 488-nm excitation of green fluorescent protein and DsRed and VLD excitation of CFP was therefore feasible and was demonstrated. Similar levels of excitation efficiency between krypton-ion and VLD sources also were observed for ELF-97 detection.

Conclusions: These evaluations confirmed that VLDs may be cost- and maintenance-effective replacements for water-cooled gas lasers for applications requiring violet excitation in addition to DNA binding dyes. *Cytometry Part A* 54A:48–55, 2003. Published 2003 Wiley-Liss, Inc.[†]

Key terms: violet laser diode; Cascade Blue; Pacific Blue; cyan fluorescent protein; 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazoline

Flow cytometry and its allied technologies are dependent on lasers as an excitation source for the ever expanding group of fluorogenic molecules available for biological and biomedical analyses. Technologic development in flow cytometry has largely paralleled the development of laser technology. Water-cooled gas lasers have been a traditional choice for fluorochrome excitation in flow cytometry for almost 30 years; they possess extremely low noise levels, stable power levels, and true TEM₀₀ beam configurations. Their high power levels have allowed useful fluorochrome detection on the fluorescence-activated cell sorter, where the ability to physically sort cells has required a compromise of poorer light-collecting optics. Their power levels also have provided practical emission levels for a larger group of laser emission lines that possess relatively weak emission energies. For some of these wavelengths (e.g., ultraviolet [UV], near-UV, and violet), they have traditionally represented the only

practical option for flow cytometric applications. The plethora of fluorescent probes now available to biomedical investigators for flow cytometry requires the availability of a wide variety of excitation wavelengths. In the UV and near-UV ranges, for example, important probes such as the calcium chelator indo-1, the Hoechst and 4,6-diamidino-2-phenylindole (DAPI) DNA binding dyes, and the expressible fluorescent proteins cyan fluorescent protein (CFP) and blue fluorescent protein require UV or near-UV/violet excitation.

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cyto.a.10046

Nevertheless, these lasers possess a number of disadvantages, including great expense in initial purchase and maintenance, a requirement for large volumes of cooling water, and considerable size and weight. Air-cooled gas lasers (including argon, helium-cadmium, and helium-neon) have become common on flow cytometers in recent years due to their lower cost, smaller size, and simpler electrical and cooling requirements. Nevertheless, they are currently limited in their useful laser wavelengths. Low power argon-ion lasers usually produce useful laser light only in their most powerful emission lines (488 and 514.5 nm), which do not include the UV or violet range. Helium-neon lasers with higher power ratings have been developed almost exclusively for 633-nm red emission, with other wavelengths only built with lower power ratings and seeing limited use in flow cytometry. Helium-cadmium lasers emit in the UV (325 nm) and blue (442 nm) ranges but remain large and expensive; they also develop considerable levels of laser "noise" over time (1).

Solid-state lasers, in particular diodes, are becoming more prevalent in fluorescence analysis instrumentation, including flow cytometers. These lasers are small and inexpensive, have low electrical and cooling requirements, and are increasingly reliable for long-term use (1). Red laser diodes now are used extensively in flow cytometers. Diode-pumped solid-state 532-nm green lasers have been incorporated into several small flow cytometers. Recently developed diode-pumped solid-state lasers emitting at 488 nm may one day replace argon-ion lasers in many instruments. The gallium nitride/indium gallium nitride UV and violet laser diodes (VLDs) recently developed by Nakamura and colleagues (2) have opened up the possibility of placing small, low-cost, and low-maintenance lasers in flow cytometers that cover the near-UV to violet excitation spectrum, thereby allowing the use of fluorochromes previously excitable only by more expensive gas lasers. Shapiro and colleagues recently incorporated a VLD into a flow cytometer and were able to carry out DNA content analysis with the UV-excited DNA probes DAPI and Hoechst 34580 with precisions approaching those of gas lasers (3). In this study we evaluated the ability of VLDs to excite a variety of important violet-excited fluorescent probes important to biological and biomedical research, including the phenotyping fluorochromes Cascade Blue and Pacific Blue, the expressible fluorescent protein CFP, and the fluorogenic alkaline phosphatase (AP) substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazoline (ELF-97). This study and recent commercial efforts to incorporate violet lasers into flow cytometric instrumentation suggest that these laser sources will be useful and cost-effective alternatives to traditional water-cooled gas lasers.

MATERIALS AND METHODS

Violet Laser Diode

These evaluations and comparisons used a single transverse mode VLD with circularization optics and built-in

Peltier temperature control (Power Technology, Inc., Alexander, AR). Beam shape was circular to within 1.2 milliradians and was approximately 2 mm in diameter. Maximum laser power was 30 mW, and maximum output post-circularization optics was 18 mW. Power level was set at 15 mW for these experiments and confirmed with a laser power meter (Melles Griot, Carlsbad, CA). The VLD temperature controller was set to maximize emission at 408 nm.

Krypton-Ion Laser

These evaluations also used a Coherent (Santa Clara, CA) I-302C krypton-ion water-cooled laser emitting at 407 or 413 nm in single-line mode. Power levels were set from 15 to 75 mW. Because internal laser power metering in higher power gas lasers becomes inaccurate at very low voltage levels, an external meter (Melles Griot, Carlsbad, CA) was used to monitor krypton laser power levels below 50 mW.

Instrumentation and Analysis Conditions

All experiments were performed on a FACSVantage SE (Becton-Dickinson [BD] Biosciences, San Diego, CA) with DiVa digital upgrade and a custom 11-color optical bench. Violet laser excitation was integrated into the cytometer in one of two ways: (i) VLD as the secondary laser on a FACSVantage SE with a Coherent I-90 argon-ion laser providing primary laser excitation and triggering or (ii) a Coherent I-302C krypton-ion laser as the secondary laser on the same FACSVantage SE with the same primary laser source. The FACSVantage SE used a standard 50-mm focal length laser focusing lens and a 70- μ m stream-in-air nozzle. Cascade Blue was detected through a 440/10-nm narrow bandpass filter, and Pacific Blue was optimally detected through a 463/50-nm filter sandwiched with a 488-nm notch filter; the notch filter was necessary to prevent spillover noise from the 488-nm primary laser source, particularly at low laser power. Similarly, CFP was detected with a 485/22-nm filter also sandwiched with a 488-nm notch filter, and green fluorescent protein (GFP) and DsRed were detected through 530/30- and 610/20-nm filters respectively. ELF-97 alcohol was detected with a 535/45-nm filter. Data was acquired with DiVa digital acquisition software (BD Biosciences) and analyzed with WinMDI 2.8 (Dr. Joseph Trotter, Scripps Institute, and BD Biosciences). Software compensation was carried out with WinList 4.0 (Verity Software House, Topsham, ME). All comparisons were made from the same samples on the same day, with the same detector voltage settings, when possible (except where noted). In some cases, photomultiplier (PMT) voltage was increased by a fixed amount to bring the lower background autofluorescence generated by low power krypton-ion or VLD excitation onto the log scale to reduce the error inherent in low-level fluorescence measurement and to allow more accurate calculation of signal-to-noise ratio; this is noted and illustrated in the figures where appropriate. Median fluorescence intensity values for background and specific labeling and their ratios were used for approximate comparisons between

excitation sources. Day-to-day variations in laser excitation and emission detection were quality controlled with violet-excited single-intensity 2- μm green-yellow fluorescent beads (Polysciences, Harrington, PA) and InSpeck multiple-intensity 2.5- μm bead cocktails (Molecular Probes, Eugene, OR). InSpeck beads were also used to monitor PMT detection linearity.

Cells and Cell Labeling

All cell lines were obtained from the American Type Culture Collection (Manassas, VA). Immunophenotyping experiments with Cascade Blue and Pacific Blue were carried out with EL4 murine thymoma cells labeled with biotin-conjugated anti-CD44 or anti-CD90 (Caltag Laboratories, Burlingame, CA) and streptavidin-conjugated Cascade Blue or Pacific Blue (Molecular Probes), followed by fixation in 1% paraformaldehyde and analysis within 24 h. Immunophenotyping with ELF-97 was also carried out in EL4 cells labeled with biotin plus anti-CD44 or anti-CD90 and streptavidin-AP (Life Technologies, Gaithersburg, MD). Cells were subsequently washed in phosphate buffered saline and labeled with ELF-97 substrate (Molecular Probes) at 2 mM in phosphate buffered saline containing 2% fetal bovine serum, followed by fixation in 1% paraformaldehyde and analysis. For CFP experiments, pECFP, pEGFP-1, and pDsRed1-1 plasmids encoding the ECFP, EGFP, and DsRed fluorescent proteins, respectively, were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). The MCIN and MGIN retroviral vectors containing the EGFP gene expressed from the retroviral long terminal repeat from a murine stem cell virus on a bicistronic transcript, which also contained a downstream neomycin phosphotransferase (*neo*) gene linked via an internal ribosome entry site from the encephalomyocarditis virus, has been described elsewhere (4). The MYIN, MCIN, and MRIN retroviral vectors containing the ECFP, EGFP, and DsRed genes, respectively, were similarly constructed by inserting the respective fluorescent protein genes into the same murine stem cell virus-based *neo*-linked internal ribosome entry site retroviral vector backbone (5,6). Stable helper-free retroviral producer lines were generated by transduction of GP+E-86 ecotropic packaging cells followed by G418 selection, as described previously (7).

RESULTS

Instrument Configuration

The purpose of this study was to determine whether some commonly used violet-excited fluorescent molecules could be detected on a flow cytometer equipped with a VLD and to determine whether this excitation was similar to that observed with a more powerful krypton-ion violet laser source. The prototype VLD used in these studies is shown in Figure 1a and was tuned to emit at approximately 408 nm at 15-mW power output. The laser and instrument configurations used in the study were set up on the same FACSVantage SE equipped with the DiVa digital acquisition system. The VLD was set up in the secondary position with an argon-ion laser emitting at 488

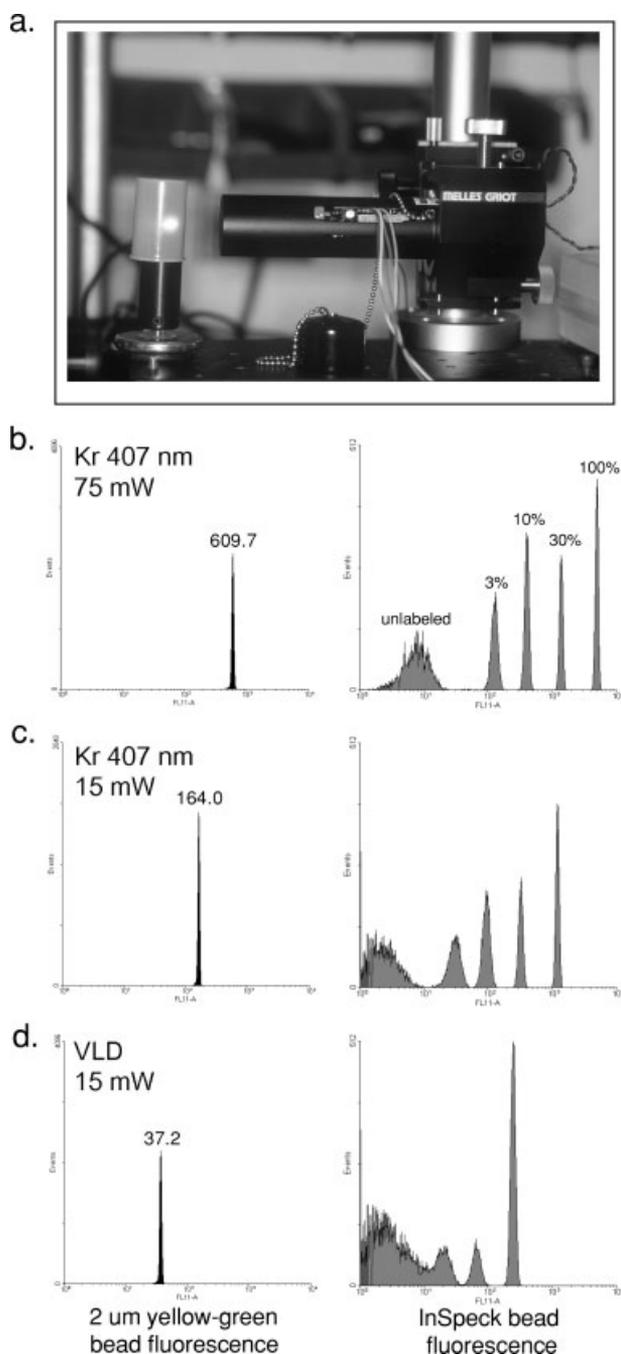
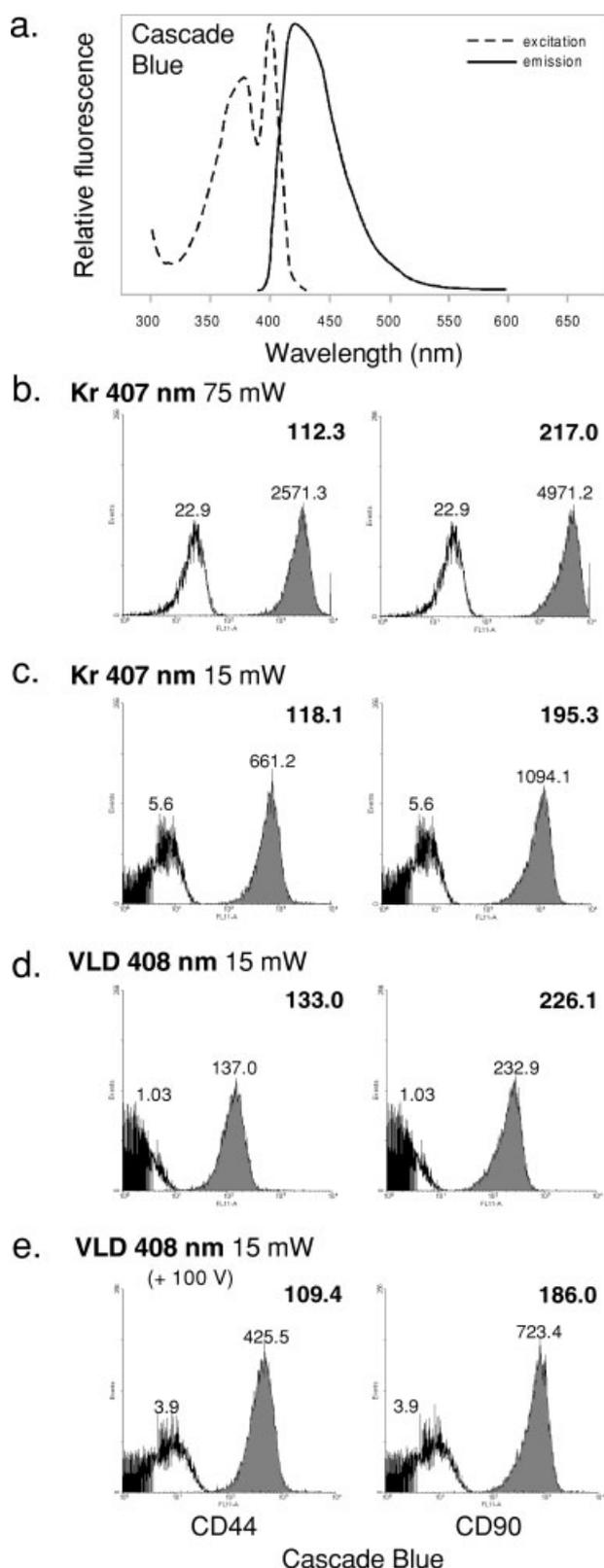


FIG. 1. Violet laser diode (VLD). **a**: The Power Technology 408-nm, 30-mW VLD used in these studies. **b**: Polyscience 2- μm green-yellow (left) and InSpeck 2.5- μm blue (right) fluorescent bead standards with Kr 407-nm, 75-mW excitation. **c**, **d**: These bead standards with Kr 407-nm, 15-mW and VLD 408-nm, 15-mW excitations, respectively, at the same photomultiplier (PMT) voltage setting as that shown in panel b. InSpeck bead fluorescence was detected through a 440/10-nm filter, and Polyscience bead fluorescence was detected through a 535/45-nm filter. Median fluorescent intensity values are shown.

nm in the primary position; for comparison, the krypton-ion source emitting at 407 nm was set up in the same position. The krypton-ion laser was set to a standard



75-mW power level or at an attenuated 15-mW level to more closely approximate the VLD power level, with external power level confirmation. Unlabeled and fluorochrome-labeled cells were analyzed for median fluorescence intensity, and the ratios were calculated between background and specific labeling. Although the large errors associated with background fluorescence measurements are a limitation on this method of comparison, calculation of the labeling ratio provides an approximate value for the signal-to-noise ratio for rough comparisons between laser sources. In cases in which the low power lasers produced a very low background value (in comparison to higher power sources), the PMT gain was increased by an indicated amount to decrease this error and provide a more accurate signal-to-noise calculation.

To initially determine the excitation efficiency of both laser sources, UV-excited Polyscience green-yellow 2- μm beads and InSpeck bead mixtures (Molecular Probes) containing 2.5- μm beads at 1%, 3%, 10%, 30%, and 100% relative intensities were analyzed with all configurations (Fig. 1b–d). Krypton at 407-nm excitation at 75 mW easily distinguished the 3% InSpeck bead population from unlabeled background, with some loss of sensitivity with reduction to 15 mW. The VLD showed a roughly 10-fold decrease in excitation efficiency and easily distinguished only the 10% relative population from background. Analysis of green-yellow beads showed a similar relative decrease in excitation efficiency. Nevertheless, these commercial bead preparations were not optimally excited in the violet range. Analysis of violet-excited fluorochromes produced much closer values between excitation sources (below).

Cascade Blue and Pacific Blue

Cascade Blue and Pacific Blue are low-molecular-weight fluorochromes developed by Haugland and colleagues (8) and are optimally excited by violet laser lines (9). Their excitation and emission spectra are shown in Figures 2a and 3a. They have proven useful in polychromatic flow cytometry due to their relative brightness (approximating fluorescein) and their red-shifted excitation maxima, thus avoiding much of the autofluorescence associated with UV-excited fluorochromes such as the coumarins (10–12). Krypton-ion excitation at 407 or 413 nm is traditionally used to excite these fluorochromes. This is shown for

FIG. 2. Violet laser diode (VLD) excitation of Cascade Blue. **a:** Excitation and emission spectra for Cascade Blue. Excitation spectral traces are dotted, and emission traces are solid. All curves are normalized. **b–e:** Flow cytometric detection of Cascade Blue. Mouse EL4 cells were labeled with biotin plus anti-CD44 (left column) or anti-CD90 (right column), followed by streptavidin conjugated to Cascade Blue, and analyzed with (b) the 407-nm, 75-mW krypton-ion laser as a secondary source, (c) the 407-nm, 15-mW krypton-ion laser as the secondary source, (d) the 408-nm, 15-mW VLD, and (e) the 408-nm, 15-mW VLD with photomultiplier (PMT) amplifier voltage increased by 100 V. All samples were analyzed on the same instrument, on the same day, and at the same PMT voltage level (with the exception of e), with a 440/10-nm filter. Specific labeling (solid areas) and background autofluorescence (open areas) peaks are shown. Median fluorescence intensity values are shown for each peak at the top of each histogram, with the ratio in boldface.

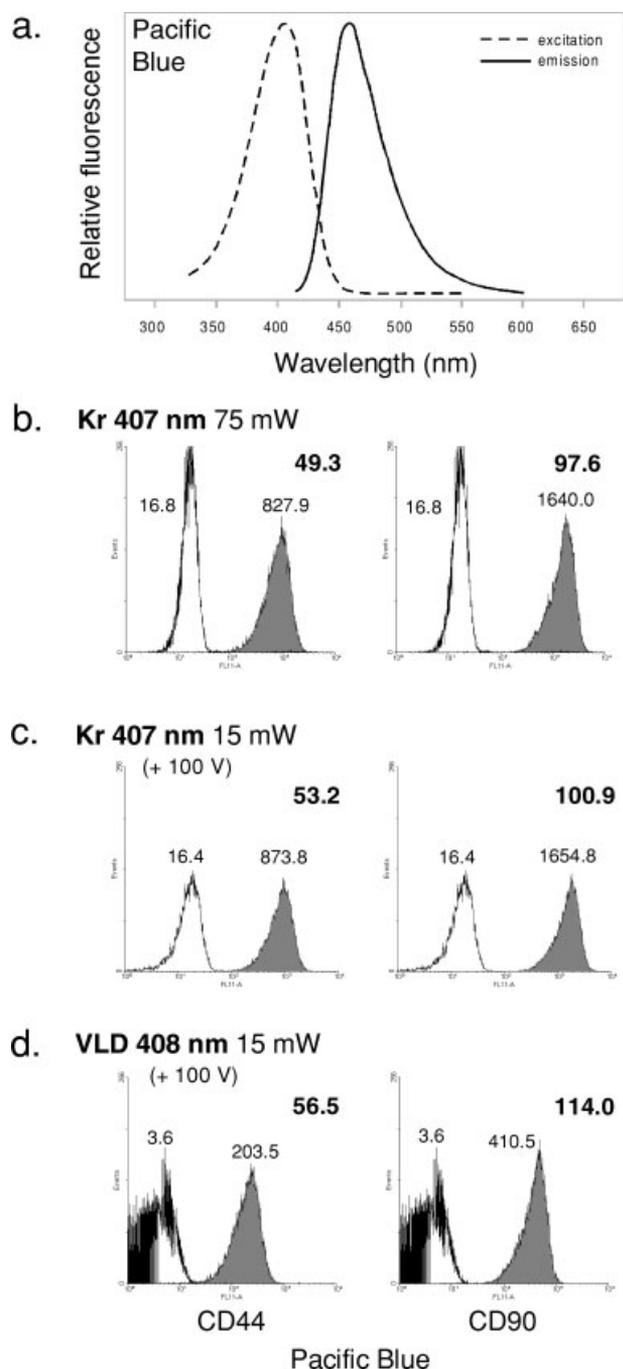


FIG. 3. Violet laser diode (VLD) excitation of Pacific Blue. **a**: Excitation and emission spectra for Pacific Blue. Excitation spectral traces are dotted, and emission traces are solid. All curves are normalized. **b–d**: Flow cytometric detection of Pacific Blue. Mouse EL4 cells were labeled with biotin plus anti-CD44 (left column) or anti-CD90 (right column), followed by streptavidin conjugated to Pacific Blue, and analyzed with (b) the 407-nm, 75-mW krypton-ion laser as the secondary source, (c) the 407-nm, 15-mW krypton-ion laser with photomultiplier (PMT) amplifier voltage increased by 100 V, and (d) the 408-nm, 15-mW VLD with PMT amplifier voltage increased by 100 V (same as c). All samples were analyzed on the same instrument, on the same day, and with a 463/50-nm filter sandwiched with a 488-nm notch filter. Specific labeling (solid areas) and background autofluorescence (open areas) peaks are shown. Median fluorescence intensity values are shown for each peak at the top of each histogram, with the ratio in boldface.

Cascade Blue in Figure 2b and 2c, where Cascade Blue CD44- and CD90-labeled EL4 cells were excited with krypton-ion at 407 nm and at laser powers of 75 mW (Fig. 2b) and attenuated to 15 mW (Fig. 2c) at equivalent detector voltages. Although the higher laser power level resulted in a higher background signal (and higher raw fluorescence value for specific labeling), Cascade Blue showed similar signal-to-noise ratios and sensitivities at high and low power krypton-ion laser excitations. Excitation of Cascade Blue with the VLD produced levels of detection comparable with those of the attenuated krypton-ion laser (Fig. 2d and 2e). Similar results were obtained with Pacific Blue (Fig. 3b–d).

Cyan Fluorescent Protein

CFP is an *Aequorea victoria*-derived fluorescent protein that is important as a single expressible fluorescent marker, a component of multicolor fluorescent protein protocols, and a donor fluorochrome for fluorescence resonance energy transfer experiments (13,14). The excitation and emission spectra for CFP are shown in Figure 4a. CFP is optimally excited by violet to blue, 420- to 440-nm, light, a spectral area with little coverage by gas and solid-state lasers typically found on flow cytometers. Most investigators use the violet krypton-ion lines or the blue 457-nm line found on argon-ion lasers or, less commonly, a 442-nm helium-cadmium laser, all of which produce reasonable CFP excitation (14,15). Krypton-ion violet excitation of CFP is shown in Figure 4b and 4c, where CFP-expressing 3T3 cells were excited at 407 nm at laser powers of 75 mW (Fig. 4b) and attenuated to 15 mW (Fig. 4c). CFP appeared to be excited equally well with higher and lower krypton-ion laser power levels. Once again, the VLD produced acceptable levels of detection sensitivity for CFP when compared with high power and voltage-matched krypton-ion excitations after accounting for reduced autofluorescence background (Fig. 3d and 3e). More importantly, VLD excitation of CFP was compatible with other fluorescent proteins in multicolor experiments (15–17). Figure 3e shows the analysis of a “cocktail” of 3T3 cells expressing no fluorescent protein, GFP, DsRed, or CFP on a FACSVantage SE equipped with the VLD. The VLD was able to adequately excite CFP and function compatibly with argon-ion 488-nm excitation of GFP and DsRed in a multilaser system.

ELF-97 Fluorogenic AP Substrate

Singer et al. (18) and Haugland and Johnson (19) described the development of the fluorogenic AP substrate ELF-97. Although this substrate itself is water soluble and essentially non-fluorescent, hydrolysis by AP yields a UV-excited, highly fluorescent yellow-green alcohol precipitate. The excitation and emission spectra for ELF-97 are shown in Figure 5a. This reagent has been used to identify endogenous and antibody-linked AP in histochemical applications and, more recently, in flow cytometry (20,21). The dearth of UV-excited immunophenotyping fluorochromes and its relative brightness make ELF-97 an important potential fluorescent probe for cell labeling. ELF-97

labeling of EL4 cells labeled with AP-conjugated antibodies is shown in Figure 5b–e, left and middle columns; ELF-97 labeling of UMR-106 rat osteosarcoma cells, which express high levels of endogenous AP, are shown in the right column. ELF-97 was optimally excited by UV wavelengths and adequately and equally excited by krypton-ion violet laser lines at 100- and 15-mW power levels (Fig. 5b and 5c). VLD excitation produced roughly comparable detection sensitivity to all krypton-ion laser power levels

(Fig. 5d). It would be expected that lower VLD wavelengths (currently as low as 385 nm in production instruments) would provide even better excitation.

DISCUSSION

In these studies, a low power VLD was evaluated for the excitation of violet-excited fluorescent molecules commonly used in flow cytometry. This evaluation was done with simultaneous comparison with a higher power water-cooled krypton-ion laser for excitation of commonly used violet-excited fluorochromes by flow cytometry. The VLD was tested as part of a dual-laser configuration on the same FACSVantage SE stream-in-air cell sorter. Comparisons of this type are semiquantitative at best and can be negatively influenced by many factors, including the noise level of the laser and detector linearity. Nevertheless, VLD excitations of Cascade Blue, Pacific Blue, CFP, and ELF-97 produced signal-to-noise ratios approximating those found with high and VLD-matched power levels of a krypton-ion laser source. These results indicated that VLD excitation may be a viable replacement for traditional gas lasers in applications requiring violet excitation. The availability of a range of wavelengths from violet diodes (385–440 nm) increases their value as flow cytometry laser sources for a variety of fluorescent probes.

The VLD system evaluated in this study was incorporated into a stream-in-air flow cytometer, where cells are physically separated into distinct subpopulations. It should be noted that a minor limitation in the use of diode lasers for flow cytometry has been their irregular beam shape; diode laser beams frequently assume multimodal configurations, making their incorporation into flow cytometer systems designed for lasers with true TEM₀₀ beam configurations difficult. The use of paired cylindrical lenses to render a circular beam pattern has been a requirement for many diode laser sources, a beam shaping requirement that significantly reduces laser output and frequently results in a large beam diameter and irregular beam spot. The requirement of stream-in-air sorting and analysis systems for symmetrical beam geometry is less stringent than those for quartz flow cell systems (which include virtually all benchtop flow cytometers such as the

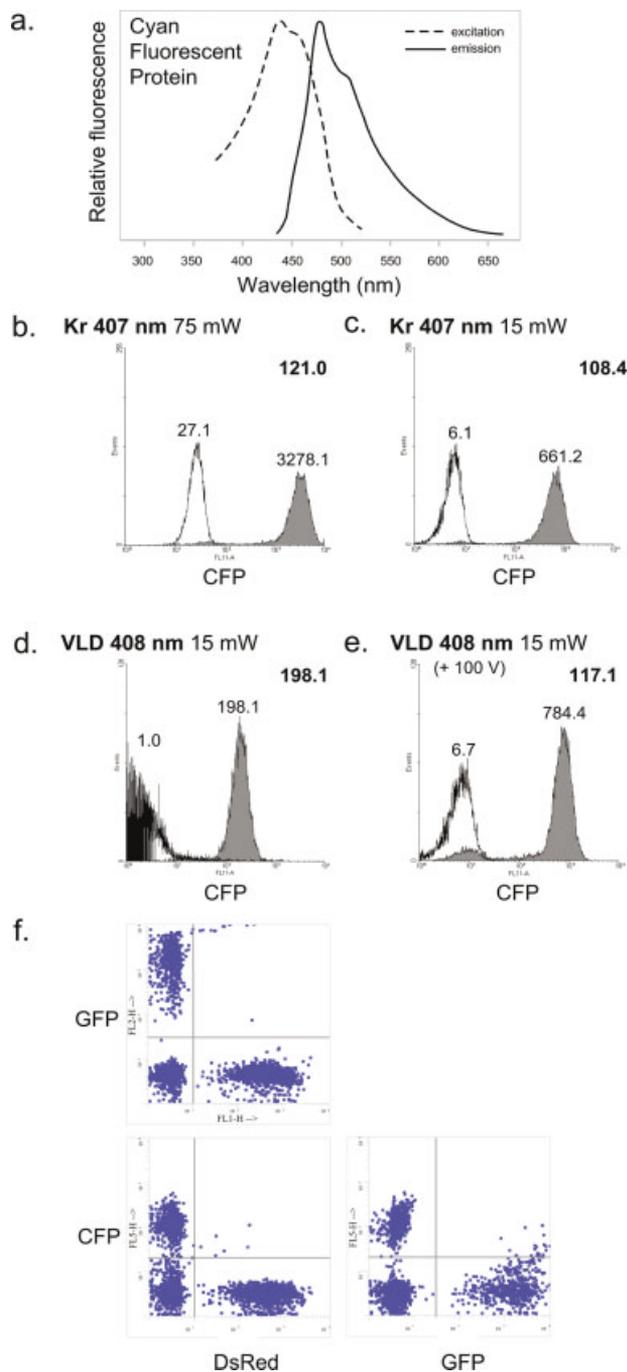


FIG. 4. Violet laser diode (VLD) excitation of cyan fluorescent protein (CFP). **a:** Excitation and emission spectra for CFP. Excitation spectral traces are dotted, and emission traces are solid; curves are normalized. **b–e:** Flow cytometric detection of CFP. Mouse NIH3T3 cells expressing CFP were analyzed by (b) the 407-nm, 75-mW laser as the secondary source, (c) the 407-nm, 15-mW krypton-ion laser, (d) the 408-nm, 15-mW VLD, and (e) the 408-nm, 15-mW VLD with photomultiplier (PMT) amplifier voltage increased by 100 V. All samples were analyzed on the same instrument, on the same day, and at the same PMT voltage level (except for e), with a 485/22-nm filter sandwiched with a 488-nm notch filter. Specific labeling (solid areas) and background autofluorescence (open areas) peaks are shown. Median fluorescence intensity values are shown for each peak at the top of each histogram, with the ratio in boldface. **f:** “Cocktail” of NIH3T3 cells expressing empty vector, enhanced green fluorescent protein (GFP), CFP, or DsRed analyzed on a FACSVantage SE with 408-nm, 15-mW VLD in the secondary laser position. Data were software compensated with WinList 4.0 (Verity). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

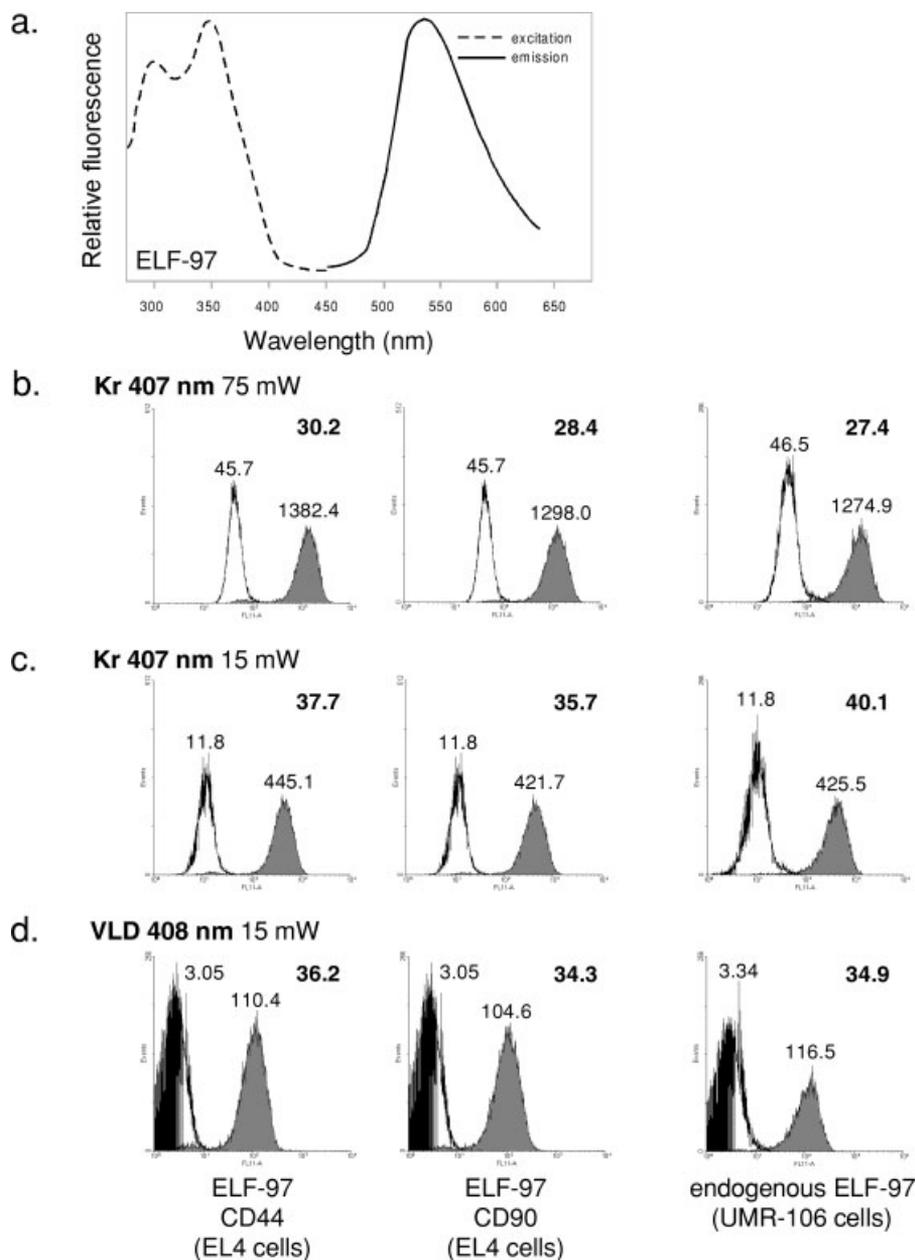


Fig. 5. Violet laser diode (VLD) excitation of 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazoline (ELF-97) alkaline phosphatase (AP) substrate. **a:** Excitation and emission spectra for ELF-97. Excitation spectral traces are dotted, emission traces are solid, and curves are normalized. **b–d:** Flow cytometric detection of ELF-97. Mouse EL4 cells were labeled with biotin plus anti-CD44 or anti-CD90, followed by streptavidin-conjugated AP, and ELF-97 substrate (left and middle columns). UMR-106 rat osteosarcoma cells expressing high levels of endogenous AP were also labeled with ELF-97 substrate (right column). Cells were then analyzed by (b) the 407-nm, 75-mW laser as the secondary source, (c) the 407-nm, 15-mW VLD, and (d) the 408-nm, 15-mW VLD. All samples were analyzed on the same instrument, on the same day, at the same photomultiplier voltage level, with a 535/45 nm filter. Specific labeling (solid areas) and background autofluorescence (open areas) peaks are shown. Median fluorescence intensity values are shown for each peak at the top of each histogram, with the ratio in boldface.

FACSCalibur and the Beckman Coulter XL). Instrument manufacturers incorporating violet diodes into benchtop flow cytometers are preferentially incorporating laser diodes with uniform beam profiles or are using fiber optic collimation systems to further refine beam shape. This limitation should be kept in mind when integrating all diode lasers (including the VLD) into flow cytometric systems with enclosed flow cells.

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