

# Analysis of UV-Excited Fluorochromes by Flow Cytometry Using Near-Ultraviolet Laser Diodes

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**Introduction:** Violet laser diodes have become common and reliable laser sources for benchtop flow cytometers. While these lasers are very useful for a variety of violet and some ultraviolet-excited fluorochromes (e.g., DAPI), they do not efficiently excite most UV-stimulated probes. In this study, the next generation of InGaN near-UV laser diodes (NUVLDs) emitting in the 370–375-nm range have been evaluated as laser sources for cuvette-based flow cytometers.

**Methods:** Several NUVLDs, ranging in wavelength from 370 to 374 nm and in power level from 1.5 to 10 mW, were mounted on a BD Biosciences LSR II and evaluated for their ability to excite cells labeled with the UV DNA binding dye DAPI, several UV phenotyping fluorochromes (including Alexa Fluor 350, Marina Blue, and quantum dots), and the fluorescent calcium chelator indo-1.

**Results:** NUVLDs at the 8–10-mW power range gave detection sensitivity levels comparable to more powerful solid-state and ion laser sources, using low-fluorescence

microsphere beads as measurement standards. NUVLDs at all tested power levels allowed extremely high-resolution DAPI cell cycle analysis, and sources in the 8–10-mW power range excited Alexa Fluor 350, Marina Blue, and a variety of quantum dots at virtually the same signal-to-noise ratios as more powerful UV sources.

**Conclusions:** These evaluations indicate that near-UV laser diodes installed on a cuvette-based flow cytometer performed nearly as well as more powerful solid-state UV lasers on the same instrumentation, and comparably to more powerful ion lasers on a jet-in-air system, and. Despite their limited power, integration of these small and inexpensive lasers into benchtop flow cytometers should allow the use of flow cytometric applications requiring UV excitation on a wide variety of instruments. © 2004 Wiley-Liss, Inc.

**Key terms:** solid-state laser; near-UV laser diode; Nd-YAG

Flow cytometers are dependent on lasers as excitation sources for an expanding group of fluorogenic molecules available for biological analysis. The ready availability of certain laser wavelengths has, to a large extent, dictated development trends in fluorescent probe technology; for example, the abundance and quality of ion laser excitation ranging from the blue-green to red wavelengths has generated intensive development of fluorescein and its derivatives, the phycobiliproteins, and cyanin dyes as probes for flow cytometry (1,2). Nevertheless, several biologically important fluorescent probes are in common and continuous use, requiring laser wavelengths less easy to obtain than the usual visible lines. The most prominent examples of this situation are the ultraviolet (UV)-excited fluorochromes, including the DNA binding dyes DAPI and the Hoechst dyes, the low-molecular-weight immunophenotyping fluorochrome aminomethylcoumarin (AMCA), and the fluorescent calcium chelator indo-1. These probes have a number of exceptional properties that are not easily replaceable by visible wavelength excited probes. For example, the ratiometric fluorescence properties of

indo-1 make it an excellent choice for semi-quantitative calcium concentration determination in live cells. Its biphasic emission profile allows calcium measurement independent of the probe loading concentration. This property has not been replicated in any single visible laser-excited calcium probe. The DNA binding dye DAPI has low nonspecific binding properties to single- and double-stranded RNA, making it a more useful cell cycle analysis probe than propidium iodide.

While there are a number of useful (and often indispensable) UV-excited fluorescent probes, equipping a

Abbreviations used: UV, ultraviolet; NUVLD, near-UV laser diode; Nd-YAG, neodymium yttrium-arsenide-garnet; FBS, fetal bovine serum; MEM, minimal essential medium; DMSO, dimethylsulfoxide.

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flow cytometer with a UV-emitting laser has always been problematic.

Water-cooled argon and krypton ion lasers have, until recently, been one of the only choices for UV excitation in flow cytometry; indeed, their extremely low noise levels, stable power levels, and true TEM<sub>00</sub> Gaussian beam configurations continue to make them very useful sources of UV light. The drawbacks of water-cooled ion sources are their size, high cost, and intensive utility and maintenance requirements, particularly when they are operated continuously in the UV (3). It is also difficult to integrate these large lasers into smaller benchtop flow cytometers (3). Until recently, there were no adequate alternatives for water-cooled sources. Helium-cadmium lasers that emit at 325 nm have been in use in flow cytometry for many years; however, these lasers have significant problems in laser noise buildup that have never been entirely resolved, and are still relatively large and maintenance-intensive (4). Mercury arc lamp equipped flow cytometers, such as the Partec PAS and the RATCOM/NPE Analyzer, generate UV lines useful for cell cycle analysis with DNA probes such as DAPI, but the excitation power available from such instruments is marginal for dimmer probes such as the coumarin fluors and indo-1. As a result, most UV applications in flow have generally been restricted to instruments capable of supporting large water-cooled ion lasers, generally a jet-in-air cell sorter (e.g., the BD FACSVantage, Beckman Coulter Altra, or Cytomation MoFlo). While a necessity when sorting is desired, the low light collection efficiency of jet-in-air optics can pose a problem for UV applications requiring high resolution or sensitivity, such as cell cycle, immunophenotyping, or calcium flux measurement.

Two promising developments in laser development, however, have addressed the problem of a producing a small inexpensive UV laser source. One is the development of the frequency tripled Nd-YAG laser, which produces a very useful line at 355 nm (5). These lasers were originally developed for stereolithography and industrial manufacturing; development is ongoing for lasers operating at power levels useful for flow cytometry (20–350 mW), and commercially available lasers suitable for flow cytometry are beginning to appear. The high-power levels from these pumped laser sources are will make them useful for both cuvette and jet-in-air systems; unfortunately, they are relatively expensive and still somewhat large for small instrument integration. The second major development is in the area of laser diodes (6). The wavelength of these small, reliable laser sources has gradually decreased, with violet sources in the 395–41-nm range becoming commonplace on commercial instruments (7–11). More recently, reliable diodes in the 370–375-nm range have been produced, and the power levels are reaching levels useful for flow cytometry (>5 mW) (11). While this power level is probably insufficient for jet-in-air instruments, it should be quite suitable for flow cytometers equipped with quartz cuvettes. As with previous diode lasers, incorporation of anamorphic optics allows the generation of beam shapes suitable for flow cytometry,

allowing laser diodes to produce beam shapes approximating TEM<sub>00</sub> configurations (11). Although the wavelengths of these lasers are somewhat longer than those traditionally used for flow cytometry, most of the important UV reagents should theoretically excite well by this range. In this study, we have evaluated several near-UV laser diodes (NUVLDs) on a cuvette-based flow cytometer for their ability to analyze a variety of UV-excited probes, including DAPI, the AMCA-derived immunophenotyping fluorochromes Alexa Fluor 350 and Marina Blue, quantum dots and the calcium chelator indo-1. For most of these applications the NUVLD sources at higher power levels (>5 mW) proved very useful for probe excitation, supporting their utility as small, inexpensive laser sources for cuvette-equipped flow cytometers.

## MATERIALS AND METHODS

### Cells and Standards

EL4 thymoma cells were obtained from the ATCC (Manassas, VA) and passaged in complete RPMI-1640 media (containing 10% fetal bovine serum [FBS] supplemented with *l*-glutamine and penicillin/streptomycin). Chicken red blood cells (CRBCs) and calf thymus nuclei (CTNs) were obtained as formaldehyde-fixed preparations from BD Biosciences (San Jose, CA). Trout red blood cells (TRBCs) were obtained from NPE Systems (Pembroke Falls, FL). Two micron yellow-green polymer microspheres (Polyscience, Harrington, PA) were used as a standard for UV and near-UV laser alignment. InSpeck Blue linearity bead arrays (Molecular Probes, Eugene, OR) and Spherotech 8-population rainbow beads (Spherotech, Libertyville, IL) were used to assess instrument sensitivity.

### DNA Content Analysis

CRBCs, TRBCs, and CTNs were washed and resuspended in phosphate-buffered saline (PBS) containing 0.5% Nonidet P-40 (NP-40) detergent (to generate nuclei) and the UV-excited DNA binding dye DAPI at 3 µg/ml (Molecular Probes) immediately prior to analysis. EL4 cells were washed twice with PBS and resuspended in same buffer with DAPI prior to analysis.

### Fluorescent Immunophenotyping

EL4 cells were washed once with PBS containing 2% FBS (no sodium azide) and incubated with biotin-conjugated antibody against either mouse CD44 or CD90 (Caltag Laboratories, San Diego, CA). Cells were then washed with PBS/FBS and secondary labeled with one of the following streptavidin-conjugated reagents: Alexa Fluor 350 or Marina Blue (Molecular Probes), or Qdot 525, 565, or 585 (Quantum Dot Corporation, Fremont, CA) (12). All samples were then washed again with PBS/FBS and fixed with 2% formaldehyde in PBS. All samples were analyzed within 24 h.

### Indo-1

Indo-1 cell loading and analysis was carried out as previously described (13). The fluorogenic calcium chelator

indo-1 acetoxymethyl ester was obtained from Molecular Probes and prepared as a stock solution at 1 mg/ml in dimethylsulfoxide (DMSO). EL4 cells were washed with MEM containing calcium and magnesium with 2% FBS (MEM/FBS), resuspended at  $5 \times 10^6$  cells/ml and incubated with indo-1 at 5  $\mu$ g/ml final concentration for 45 min at 37°C. Cells were then washed twice with MEM/FBS, resuspended at  $5 \times 10^6$  cells/ml, incubated for an additional 10 min at 37°C with no additional indo-1, washed two times more and finally resuspended at  $10^6$  cells/ml in MEM/FBS. Cells were kept at 37°C until analysis (within 1 h). Ionomycin at a final concentration of 1  $\mu$ g/ml was used as a calcium ionophore to induce calcium influx during analysis. DMSO alone was used as a carrier control and induced no calcium influx (data not shown).

### Flow Cytometry

Cells were then analyzed on one of two instrument configurations: (i) a BD Bioscience LSR II equipped with a frequency-tripled Nd-YAG 355-nm UV laser emitting at 20 mW (Lightwave Electronics, Mountain View, CA), or (ii) the same BD LSR II equipped with one of several near-UV laser diodes. Cells were excited on the LSR II with one of the following lasers: (i) a Power Technology (Alexander, AR) 372 mW near-UV laser diode emitting at 1.5 mW; (ii) a Power Technology 370-nm near-UV laser diode emitting at 8 mW; a (iii) Coherent (Auburn, CA) Radius 372-nm near-UV laser diode emitting at 8 mW; or (iv) a Point Source (Southampton, UK) 374-nm laser emitting at 10 mW. The Point Source laser was used without and with a coupled single mode fiber optic also manufactured by Point Source. Power levels were measured using a NIST-traceable 2W broadband power meter (400 nm to 2  $\mu$ m) with a thermopile graphite detector head from Melles Griot (Carlsbad, CA). All diode lasers were equipped with adjustable anamorphic optics, which were focused during the alignment procedure to achieve maximum sensitivity. Laser coupling to the fiber utilized high-precision three-axis gimballed mountings to both the laser-fiber junction and at the fiber output to the flow cell (Newport Instruments). Laser beam launching through the fiber optic was carried out according to the manufacturer's instructions, with an initial coarse adjustment by beam visualization and fine adjustment with the power meter. Signals derived from all lasers (Nd-YAG and diode) were aligned to the same PMTs (normally assigned to the violet diode laser on the default LSR II configuration). A full laser mirror alignment and quality control check with both Polyscience yellow-green alignment microspheres was performed following all laser mountings; bead peak CVs were <2.2 on the LSR II for all lasers prior to all experiments. DAPI, Alexa Fluor 350, and Marina Blue fluorescence were detected through a 450/50-nm narrow bandpass filter; Qdot 525 through a 525/50-nm filter; Qdot 565 through a 575/2-nm filter; and Qdot 585 through a 585/42-nm filter. The calcium-bound and -unbound indo-1 signals (violet and blue) were detected through 405/20- and 530/30-nm filters respectively, with a 505-nm longpass dichroic to split the signals. The same filters were used for all lasers. Both

flow cytometers used the FACSDiVa digital acquisition electronics and software (BD Biosciences). Data was analyzed with WinMDI version 2.8 (Joseph Trotter, BD Biosciences). For DNA content analysis, immunophenotyping fluors and quantum dots, all lasers in the 8–10-mW range performed comparably; representative data are shown for each application.

## RESULTS

### Linearity Bead Sensitivity Standards

Four NUVLD sources were evaluated in this study: (i) a Power Technology 372 mW emitting at 1.5 mW; (ii) a Power Technology 370 nm emitting at 8 mW; (iii) a Coherent Radius 372 nm emitting at 8 mW; and (iv) a Point Source 374 nm emitting at 10 mW. The Point Source NUVLD was used both without and with a coupled single mode fiber optic, which reduced the power output to ~3.3 mW. The Power Technology 8 mW, Coherent and Point Source NUVLDs mounted on the LSR II are illustrated in Figure 1a–c. After initial alignment, InSpeck Blue linearity microspheres (Molecular Probes) were analyzed to assess instrument sensitivity with the installed lasers. This microsphere system has an arbitrarily bright population ("100%"), with fractions labeled at 30%, 10%, 3%, 1%, and 0.3% of the 100% fraction. The results are shown in Figure 2a–f. Krypton-ion MLUV excitation on the on a FACS Vantage cell sorter was used as a generic baseline for comparison to the NUVLD sources (Fig. 2a). While the 1.5-mW diode gave excellent peak CVs for brightly labeled microspheres, this power level was found to be less than optimal for detection of more dimly labeled objects; the 1% labeled microsphere population was distinguishable, but the 0.3% beads could not be resolved from the unlabeled population (Fig. 2b). This was in contrast to the Power Technology, Coherent and Point Source NUVLDs emitting at 8–10 mW, which were all able to easily distinguish all microsphere populations (Fig. 2c–e). Sensitivity was at least equal and probably greater than the krypton-ion laser emitting in multiline UV mode on the FACS Vantage jet-in-air sorter, which showed somewhat less separation between the 0.3% and unlabeled microsphere fractions. Sensitivity with the Point Source laser with fiber optic coupling (3.3-mW output) was also approximately the same as the higher-power sources (Fig. 2f). This comparison verified the sensitivity of cuvette-equipped instrumentation even with low-power laser sources, and indicated that the NUVLD 8–10-mW power level should be adequate for excitation of weakly fluorescent UV probes.

The equivalence or increase in sensitivity between the krypton-ion source and the NUVLDs was in part due to the better light gathering properties of the cuvette instrumentation. Signal sensitivity and resolution of a typical NUVLD was therefore compared to a more powerful Nd-YAG 355-nm laser (20 mW), mounted on the same BD LSR II in the same position and with the signals aligned to the same PMTs. The results are shown in Figure 3. When the unlabeled and 0.3% population of InSpeck Blue microspheres

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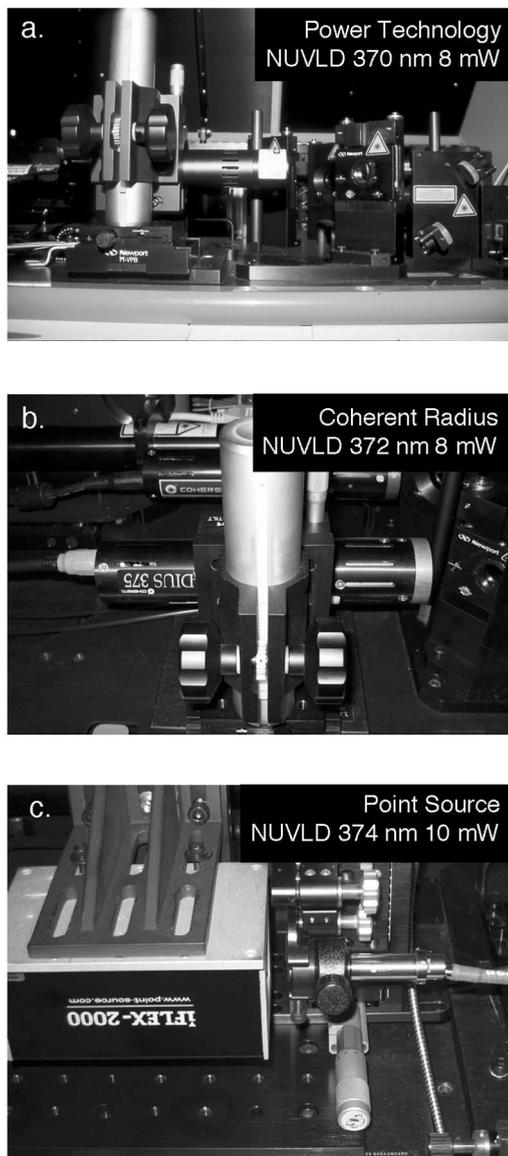


Fig. 1. Near-UV laser diodes (NUVLDs) used in this study. **a:** Power Technology NUVLD emitting at 370 nm, 8 mW. **b:** Coherent Radius emitting 372 nm, 8 mW. **c:** Point Source Ltd. NUVLD emitting at 374 nm, 10 mW, equipped with a fiber optic.

were analyzed in linear mode, the 20-mW laser give slightly better peak CVs (and hence signal resolution) than the NUVLD; however, the NUVLD results were still well within the acceptable range (Fig. 3a,b). This was further illustrated using Spherotech rainbow beads; the two dimmest bead subpopulations in this array are difficult to resolve from each other when excited by UV and detected through a blue filter, providing a good test of instrument resolution (Fig. 3c-f). Both NUVLD and Nd-YAG lasers were able to resolve these two populations, although the Nd-YAG gave slightly better discrimination (Fig. 3e,f). Although the Nd-YAG laser had somewhat better performance characteristics in this comparison, the NUVLD per-

formed at nearly the same level of signal sensitivity and resolution. The following data are representative from one of the evaluated lasers; all the 8-10-mW NUVLDs performed comparably for the indicated applications.

### DNA Cell Cycle

DNA cell cycle analysis with the UV-excited DNA binding dye DAPI represents a critical application in flow cytometry; DAPI does not bind RNA at significant levels (a problem with other DNA probes such as propidium iodide) and is generally believed to give the lowest peak CVs on any of the commonly used DNA binding reagents. Analysis of DAPI-labeled nucleated chicken red blood cells (CRBCs), a standard used for instrument calibration, using the 1.5-mW laser gave acceptable results, with CVs for the singlet population in the 2.5% range (Fig. 4a); this is typically what is achieved with propidium iodide labeling on cuvette-based flow cytometers. Moving to an 8-10-mW NUVLD, however, decreased the CRBC CV to 1.74 (Fig. 4b), and gave extremely low CVs for DAPI-labeled trout red blood cell (TRBC) and calf thymus nuclei (CTN) standards as well (Fig. 4c,d). Cell cycle analysis of DAPI-labeled EL4 cell nuclei similarly gave excellent resolution (Fig. 4e). These values are well below what is typically achieved using propidium iodide on cuvette-based flow cytometers (data not shown).

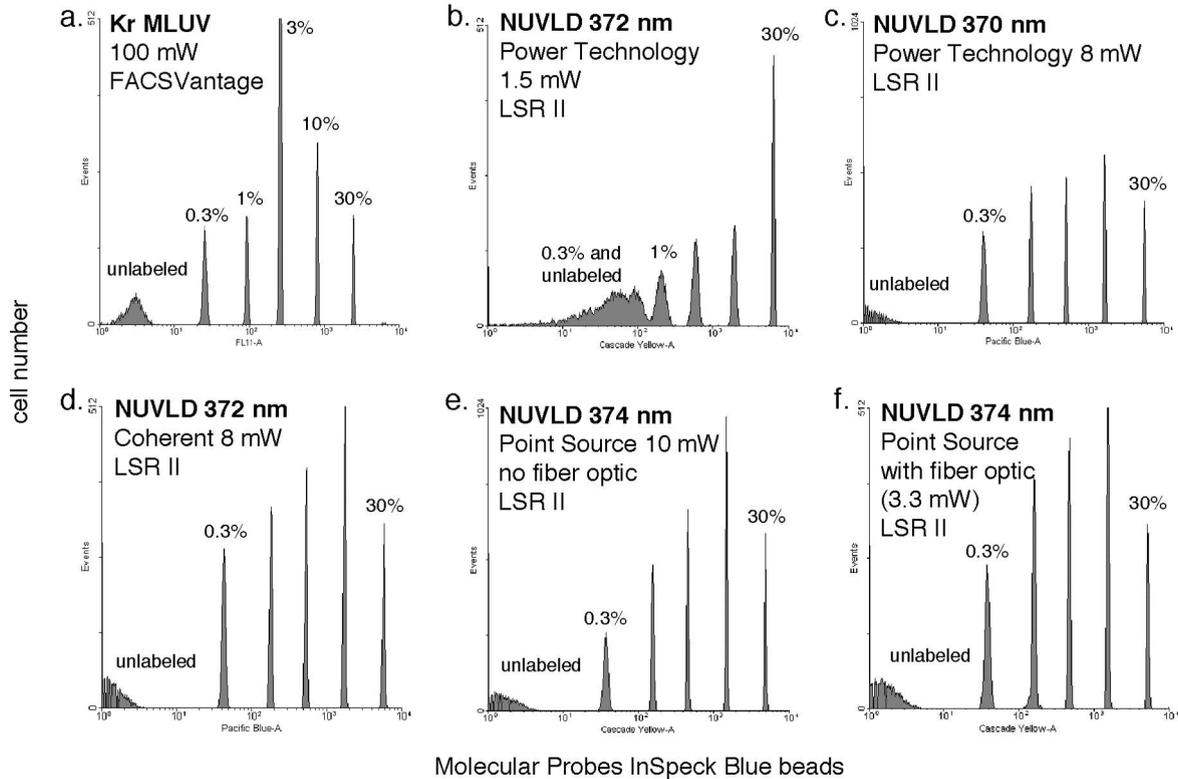
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### UV-Excited Immunophenotyping Fluorochromes

Coumarin-based low-molecular-weight fluorochromes, including 7-aminomethylcoumarin (AMCA) and the fluorinated/sulfonated and more photostable versions Marina Blue and Alexa Fluor 350 are potentially useful as fluorochromes for both extracellular and intracellular labeling. Their emission spectra are in the blue range, well-separated from fluorescein and other fluorescent probes. Nevertheless, they have seen limited use as fluors for flow cytometry. While this is part because of the high levels of autofluorescence present in the blue region of the visible spectrum (causing elevated background fluorescence and reduced signal-to-noise ratios), this is also due to the traditional presence of UV lasers on jet-in-air systems only, limiting their usage to these instruments. The poor efficiency of light-collecting optics on sorters might also be contributing to their limited usefulness as probes. To determine whether this limitation could be overcome using the combination of a NUVLD and a cuvette-based cytometer. Alexa Fluor 350 and Marina Blue labeled cells were analyzed on the BD LSR II equipped with the 8-10-mW sources). EL4 mouse thymoma cells were labeled with either biotin-anti-CD44 (a medium-density antigen) or CD90 (a highly dense antigen), followed by streptavidin conjugates of either Alexa Fluor 350 or Marina Blue, and the samples analyzed on the same LSR II with either a NUVLD or the Nd-YAG UV laser. The results are shown in Figure 4 for Alexa Fluor 350 (5a-d) and Marina Blue (Fig. 5e-h). The 8-10-mW NUVLDs gave comparable sensitivity for these probes to the more powerful solid-state laser. Analysis of Alexa Fluor 350 and Marina Blue with the 1.5-mW source on the LSR II gave

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Molecular Probes InSpeck Blue beads

FIG. 2. Sensitivity assessments of near-UV laser diodes (NUVLDs). InSpeck blue microspheres (Molecular Probes) at relative fluorescence levels of 30% (of an arbitrary 100% value), 10%, 3%, 1%, 0.3%, and unlabeled were analyzed with the indicated instrument and laser using a 450/50-nm narrow bandpass filter. **a:** Krypton-ion MLUV laser 100 mW on BD FACS Vantage DiVa. **b:** Power Technology 372-nm 1.5 mW on BD LSR II. **c:** Power Technology 370-nm 8 mW on BD LSR II. **d:** Coherent Radius 372 nm 8 mW on BD LSR II. **e:** Point Source Ltd. 374-nm 10 mW on BD LSR II. **f:** Same Point Source Ltd. 374-nm laser with fiber optic coupling (3.3-mW maximum output post-fiber).

reduced sensitivity, suggesting that these power levels are too low for the excitation of low-molecular-weight fluorophores (data not shown).

### Quantum Dots

Quantum dots (Qdots) are a class of novel fluorescent probes that are seeing increasing usage in cell biology. Quantum dots are sandwiched nanocomplexes of metal ions that emit strongly at a range of visible wavelengths depending on the composition and size of the particle; when encapsulated, these particles can be conjugated to proteins and used as fluorophores (12). Their emission bandwidths are extremely narrow, allowing good spectral separation in multicolor applications. Quantum dots have the unique property of an excitation range at almost any wavelength below their emission peak; hence, 525-nm quantum dots (emitting in the yellow-green range) can be excited at any wavelength from the ultraviolet to the blue. Their excitation efficiency peaks in the ultraviolet, however, and these reagents have been proposed for multicolor fluorescence applications using UV or violet excitation. Although primarily used at this point in epifluorescence and confocal microscopy due to their almost complete resistance to photobleaching, they have tremendous potential for flow cytometry; theoretically, five- or

six-color flow cytometry could be carried out using a single UV or violet excitation source.

Quantum dots at three emission wavelengths (525, 565, and 585 nm) were therefore analyzed using the NUVLD 8 mW lasers on the LSR II, in comparison with the Nd-YAG laser. The results are shown in Figure 6. The NUVLD lasers gave excellent excitation of all four Qdot species, at levels essentially comparable to the Nd-YAG source. As previously observed, the long-wavelength species gave greater signal-to-noise ratios than the shorter ones.

### Indo-1

The fluorescent calcium chelator indo-1, despite its need for a UV source, remains the most reliable fluorescent calcium probe for calcium flux measurement (13). Its biphasic spectral emission pattern (violet emission for the bound chelator and blue emission for unbound) allows a ratiometric analysis of calcium flux, making the measurement independent of chelator concentration in the cell. This is vastly superior to single emission chelator and chelator combinations (e.g., fluo-3 and Fura Red), which are highly dependent on concentration and chelator ratio. Most UV laser sources (including argon, krypton, helium-cadmium, and Nd-YAG) are suitable for indo-1 analysis. In

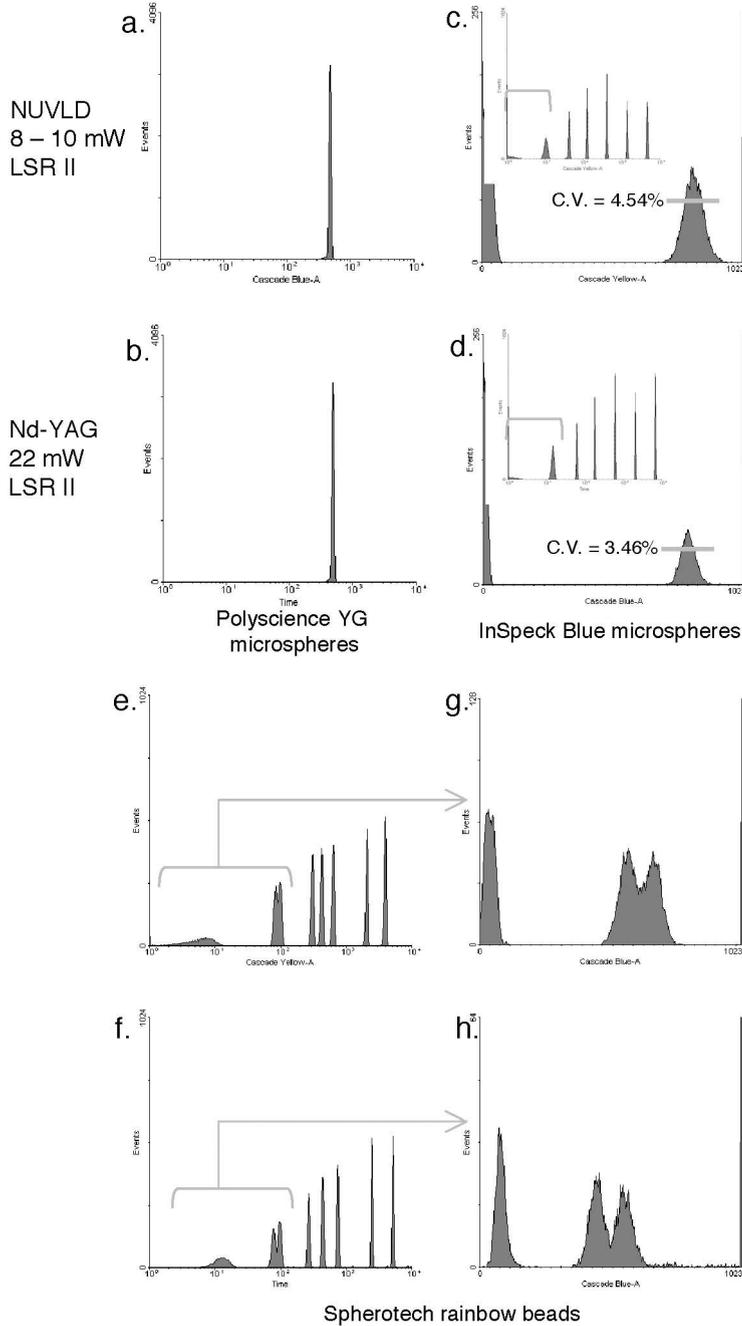


Fig. 3. Sensitivity and resolution comparison to a Nd-YAG source. **a,b**: Polyscience 2 micron yellow-green microspheres analyzed in log scaling for near-UV laser diode (NUVLD) (a) for Nd-YAG (b) on the BD LSR II. **c,d**: Unlabeled and 0.3% InSpeck Blue microsphere populations analyzed in linear scaling (full array in log scale shown in inset) for NUVLD (c) or Nd-YAG (d) on the BD LSR II. Peak CV for the 0.3% microsphere population are indicated. **e-h**: Spherotech rainbow beads analyzed in log scale (e,f), and the unlabeled and two dimmest populations analyzed in linear scale (g,h) for NUVLD (e,g) or Nd-YAG (f,h) on the BD LSR II. All microspheres were analyzed through a 450/50 narrow bandpass filter, and each microsphere type was analyzed at a fixed PMT voltage for both laser types.

F7 Figure 7, EL4 cells loaded with indo-1 at 5  $\mu\text{g/ml}$  were treated with ionomycin at 1  $\mu\text{M}$  and analyzed on the LSR II using an 8-mW near-UV diode. While the unbound calcium signal (blue 530 nm) was intense with near-UV excitation and lost immediately following ionophore treatment (Fig. 7a), no increase in bound calcium signal (violet 405 nm) above background could be distinguished (Fig. 7b). Calcium-bound indo-1 could not be detected with any of several filters, including the illustrated 405/20-nm filter with UV blocking and a sharp cutoff, and a narrow 412/13-nm filter with virtually no transmission

below 390 nm (data not shown). Similarly, the interposition of a 380 shortpass dichroic into the laser path to reduce artifactual violet fluorescence emitting from the laser head had no effect on bound indo-1 detection (data not shown). In comparison, the Nd-YAG gave excellent calcium detection on the same instrument (Fig. 7c,d).

#### DISCUSSION

A group of NUVLDs were installed on a BD LSR II cuvette-based flow cytometer and assessed for their ability

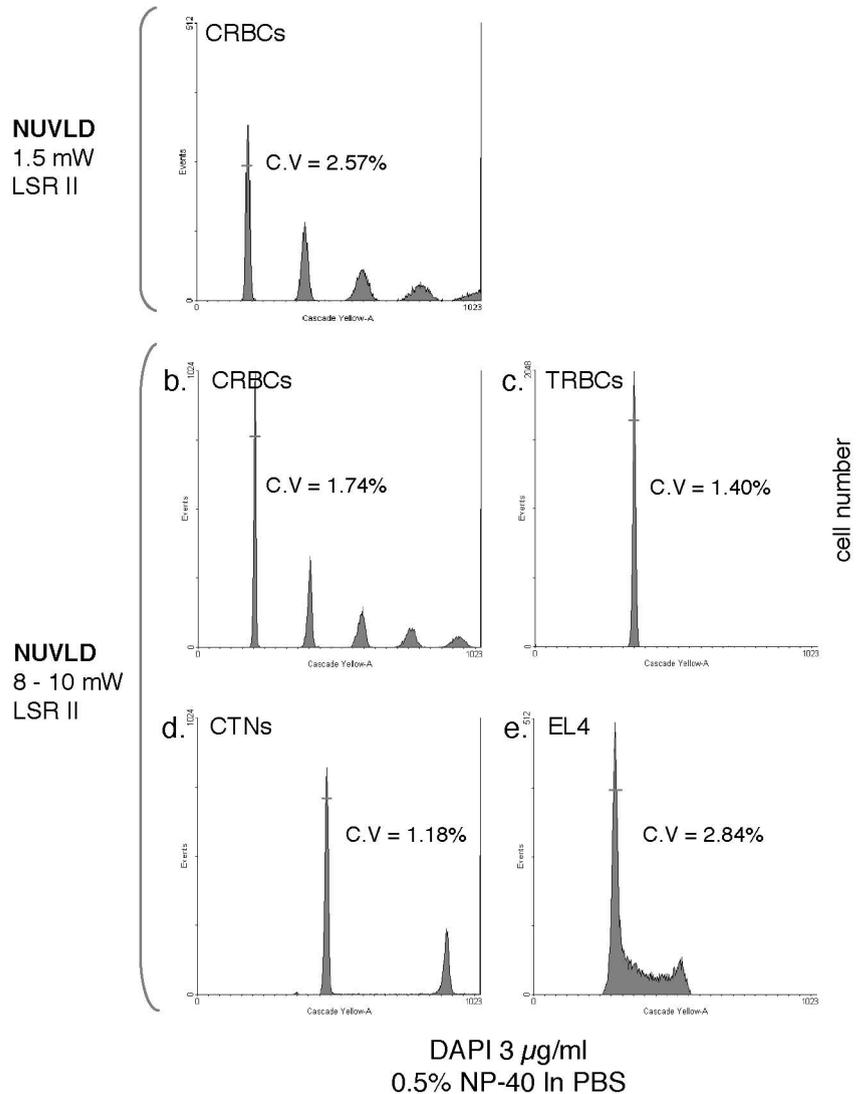


FIG. 4. DNA content analysis. DNA content standards and EL4 cell nuclei labeled with DAPI at 3  $\mu\text{g/ml}$  were analyzed on a BD LSR II equipped with either a near-UV laser diode (NUVLD) 1.5 mW (a) or 8–10 mW (b–e). a: chicken red blood cells (CRBCs) analyzed with a NUVLD 1.5 mW laser. b: CRBCs; c: trout red blood cells (TRBCs); d: calf thymus nuclei (CTNs); e: EL4 thymoma nuclei, all analyzed with a NUVLD 8–10 mW. Peak CVs are indicated on each histogram.

to excite a variety of important UV-stimulated fluorochromes. Using microsphere standards, NUVLDs performed comparably or better than far more powerful ion lasers on stream-in-air sorters, and nearly as well as more powerful solid-state UV lasers on the same cuvette instrument. DNA content analysis with DAPI gave excellent resolution with all NUVLD sources, particularly those at  $\geq 8\text{-mW}$  power levels; resolution exceeded that usually obtained with propidium iodide. Excitation of the immunophenotyping fluorochromes Alexa Fluor 350, Marina Blue, and the quantum dots with  $\pm 8\text{ mW}$  was comparable to that obtained with more powerful solid-state sources. Indo-1 could not be analyzed with NUVLDs; while this might be due in part to the close proximity of the NUVLD line (370–375 nm) to the calcium-bound signal (resulting in unacceptably high laser noise in the detector), attempts to block laser light from entering the indo-1-bound detector had no positive effect. The inability of near-UV light to efficiently excite the calcium-bound form of indo-1 may

therefore be the primary reason for this failure. In summary, NUVLD sources were able to replace traditional ion lasers for most applications requiring UV excitation, and performed at levels approaching those of more powerful solid-state UV sources.

Laser diodes (particularly red and violet) are now common fixtures on flow cytometers. However, near-UV diodes have not (at this writing) been incorporated into a commercial instrument. One of the apparent limitations is the expense and design of focusing optics that can transmit UV light; these optics are not always incorporated into less expensive benchtop cytometers. Another limitation is the recent use of fiber optics as laser light guides for flow cytometry; fiber optics cause a particularly high level of laser power loss in the UV range, and can degrade with continuous exposure to UV laser light. Nevertheless, addition of a fiber optic to a NUVLD of sufficient power still results in an adequate post-fiber power output, as illustrated in Figure 2. Higher-power NUVLDs (20 mW) that

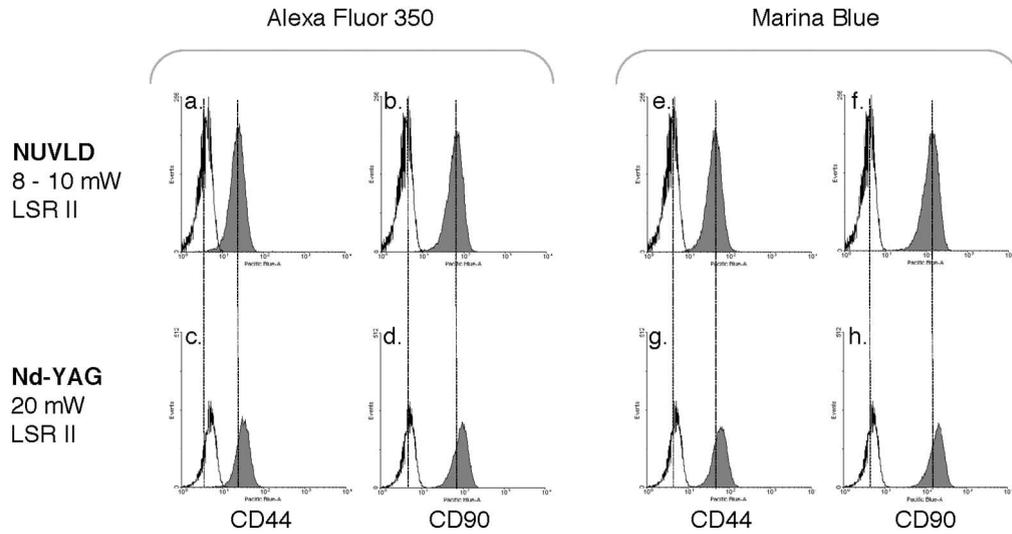


FIG. 5. Immunophenotyping fluorochrome analysis. EL4 cells were labeled with biotin-conjugated anti-CD44 or CD90 followed by streptavidin conjugates of Alexa Fluor 350 (a–d) or Marina Blue (e–h). Cells were then analyzed on a BD LSR II equipped with a near-UV laser diode (NUVLD) 8–10 mW (top row) or a Nd-YAG UV laser (bottom row).

should soon be commercially available should also be able to compensate for this power loss. It is also possible that near-UV laser light will cause less fiber degradation than frank UV. It should therefore be feasible to incorporate NUVLDs into commercial instruments in the very near future. While Nd-YAG sources are now available at power levels higher than NUVLD sources, the small size and low

cost of NUVLD sources make them attractive alternatives for UV excitation in flow cytometry.

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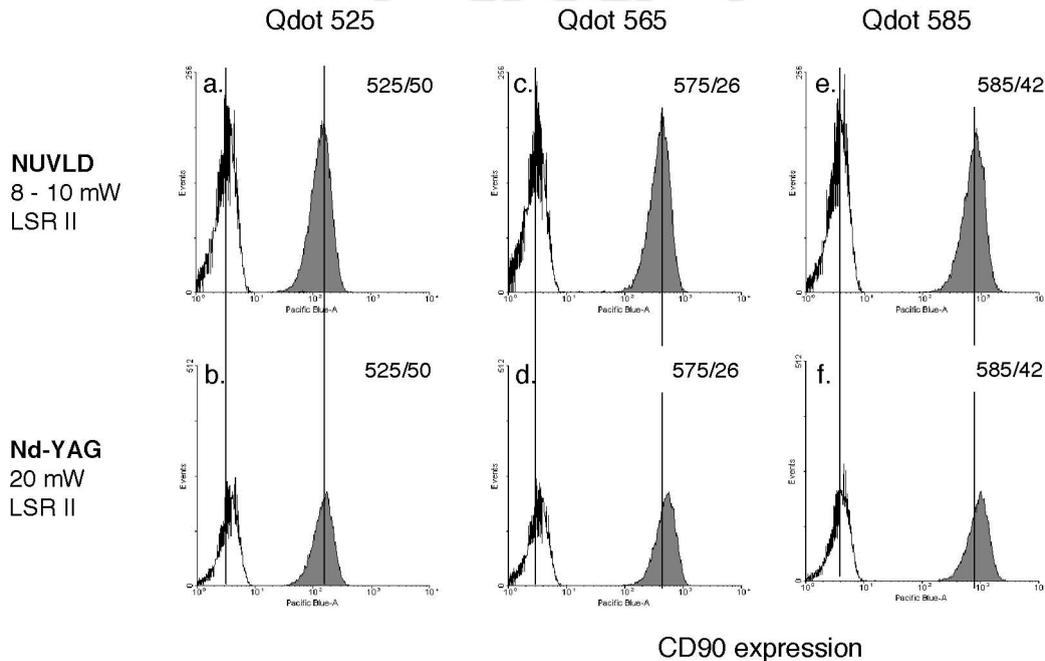
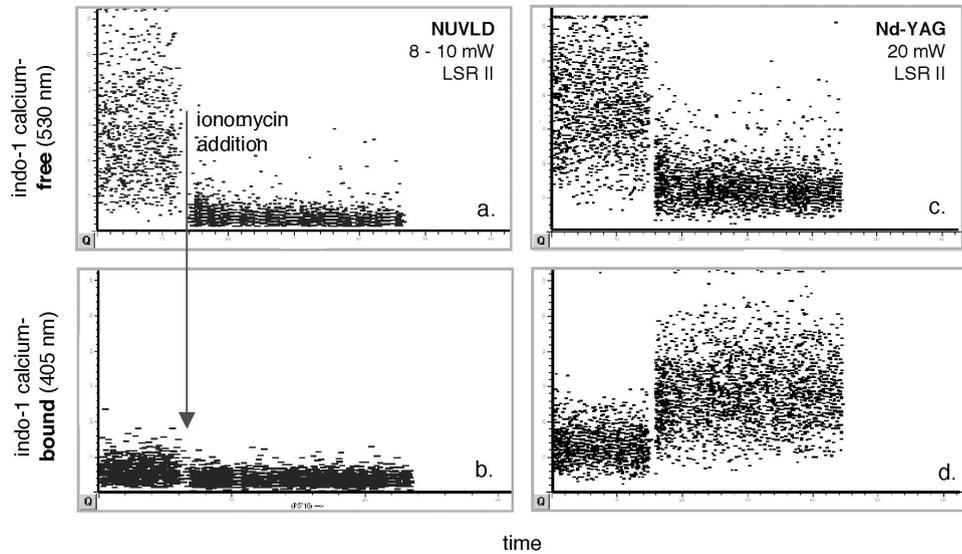


FIG. 6. Quantum dots. EL4 cells were labeled with biotin-conjugated anti-CD90 followed by streptavidin conjugates of Qdot 525 (a,b), Qdot 565 (c,d), or Qdot 585 605 (e,f). Cells were then analyzed on a BD LSR II equipped with a near-UV laser diode (NUVLD) 8–10 mW (top row) or a Nd-YAG UV laser (bottom row).

FIG. 7. Indo-1. EL4 cells were loaded with indo-1 AM ester according to the Materials and Methods, and analyzed on a BD LSR II equipped with a near-UV laser diode (NUVLD) 8–10 mW (a,b) or a Nd-YAG UV laser (c,d). Time-plotted traces are shown for the indo-1 calcium-free at 530 nm (top row) and calcium-bound at 405 nm (bottom row). Ionomycin at 1  $\mu$ M final concentration was added at 1 min following start of data acquisition (indicated by the arrow). A total of 3–3.5 min of events were collected for each analysis.



colleagues for their loan of several near-UV diodes lasers, and for valuable technical discussion.

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AQ: 1

AQ: 2

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