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# Small Lasers in Flow Cytometry

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**Summary**

Laser technology has made tremendous advances in recent years, particularly in the area of diode and diode-pumped solid state sources. Flow cytometry has been a direct beneficiary of these advances, as these small, low-maintenance, inexpensive lasers with reasonable power outputs are integrated into flow cytometers. In this chapter we review the contribution and potential of solid-state lasers to flow cytometry, and show several examples of these novel sources integrated into production flow cytometers. Technical details and critical parameters for successful application of these lasers for biomedical analysis are reviewed.

**Key Words:**

Diode, diode-pumped solid state lasers, laser.

## 1. Introduction

Flow cytometers are dependent on lasers as an excitation source for the ever-expanding group of fluorogenic molecules available for biological analysis. The choice of what lasers to use for flow cytometry has been dependent not only on available laser technology, but also on the efficiency of a flow cytometer's light collecting optics and detectors, and on the characteristics of available fluorescent probes ***(1)***.

Water-cooled gas lasers have been a traditional choice for fluorochrome excitation in flow cytometry for more than 30 yr; they possess extremely low noise levels, stable power levels, and true $TEM_{00}$ Gaussian beam configurations. The drawbacks of water-cooled gas sources are their size, their high cost, and their intensive utility and maintenance requirements. Nevertheless, they remain in daily use on many high-end flow cytometers, particularly those with jet-in-air sample systems; this is largely attributable to the low light collection efficiency of these systems, which possess numerical apertures in the area of 0.4–0.6.

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Their power levels have also given practical emission levels for a larger group of laser emission lines that possess relatively weak emission energies, such as the array of wavelengths available from krypton-ion sources. For some of these wavelengths (such as the 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.

Advances in laser, optical, and fluorochrome technology have reduced the requirements for laser power, opening up a wide variety of lower-power laser sources for flow cytometry applications. *Air-cooled gas lasers*, although similar in principle to water-cooled sources, have been integrated into smaller benchtop flow cytometers for years; these include argon-ion, helium–neon (He–Ne), and helium–cadmium (He–Cd) lasers as the most prominent examples ***(2–7)***. They have lower cooling requirements and generally require lower levels of routine maintenance. Air-cooled argon-ion lasers emitting at 488 nm are standard equipment on most benchtop analyzers, and He–Ne red 633-nm lasers make robust and long-lived sources for red-excited fluorescent probes ***(5–7)***. These lasers have lower output levels than their water-cooled predecessors; their current usefulness is also attributable to improvements in flow cytometer cuvet and light collection optics design (with numerical apertures now approaching 1.2), more sensitive photomultiplier tube detectors, and the availability of fluorochromes with greater excitation/emission efficiency.

Nevertheless, air-cooled gas lasers still possess some drawbacks, primarily high levels of heat generation. The number of available wavelengths from these low-power gas sources is also somewhat limited; air-cooled argon-ion lasers generally emit only at 488 and 514.5 nm, and He–Ne sources other than red 633 nm have traditionally been low in power and less than suitable for flow cytometric applications ***(7)***. Recent advances in solid-state laser technology have therefore been an exciting development for flow cytometry ***(8,9)***.

*Diode lasers* (in which laser light is generated by pumping electricity into a solid medium rather than a gas plasma tube) have been integrated into a number of flow cytometers. Among the first diode lasers available were the gallium aluminum arsenide (GaAlAs) variety, which emit in the infrared and formed the basis for optical disk scanning and barcode recognition systems; the development of aluminum gallium indium phosphide (AlGaInP) diodes that emit at 635 nm ultimately gave a laser useful for exciting allophycocyanin and the monomeric cyanin dyes, both useful for fluorescent immunophenotyping ***(10–12)***. Diode lasers are now dropping in wavelength; blue and violet diodes are now mass produced; violet laser diodes are now available on many benchtop flow cytometers ***(13–16)***. Near-UV diodes are at this writing (2003) in the prototyping stage, and should be practical within the year ***(17)***. These violet and near-UV lasers are

small, have minimal cooling requirements and are relatively inexpensive; they are also achieving power levels in the 5–30 mW range, quite applicable for cuvet-based flow cytometry (and even jet-in-air systems at the higher power levels).

*Diode-pumped solid-state (DPSS)* lasers are a related laser development that are also having a significant impact on flow cytometry. DPSS lasers use a diode source to pump another crystalline material (frequently neodymium yttrium aluminum garnet, or ND-YAG). Frequency doubling or tripling of the pumped laser output can produce laser wavelengths of considerable value in flow cytometry. DPSS 532-nm green lasers are being integrated into a number of instruments, including the Guava Technologies analyzer and the Luminex multiplex bead analyzer; this wavelength excites phycoerythrin (PE) with considerable efficiency. Frequency tripled lasers emitting at 355 nm, originally developed for metal fabrication, are now being reduced in power as air-cooled sources of UV laser light. DPSS 488 nm lasers are now available on several benchtop flow cytometers, including the BD LSR II and the Cytomation CyAn; these lasers emit in the 20-mW range (the same as an air-cooled argon-ion laser) but occupy a fraction of the size and cooling requirements. As for diodes, DPSS lasers are becoming available in an increasing number of wavelengths, many of which may be useful for flow cytometry.

Small, low-power gas and solid-state lasers have therefore found considerable application in flow cytometry, frequently replacing unwieldy water-cooled gas sources. Although low power levels and a lack of laser lines were early problems with these sources, improved instrument sensitivity, better fluorochromes, and an increased array of available laser wavelengths have addressed many of these concerns. In this chapter, several of these small laser sources are evaluated for their applicability in flow cytometry, with comparison to traditional laser sources wherever possible. Information for setting up these laser sources on production flow cytometers will also be provided. The following systems are examined:

*He–Ne green 543- and yellow 594-nm lasers.* Red He–Ne lasers have found extensive use in flow cytometry; they operate at low temperatures, are extremely reliable, and have easily controlled beam geometries ***(5–7)***. He–Ne laser have also been available at other wavelengths, notably green (543 nm), yellow (594 nm), and orange (612 nm); however, generation of these lines is generally inefficient, with the result being very low laser power levels (usually 1–3 mW). These low power levels have made these lasers uncommon sources in flow cytometry. Recent advances in light collection optics, as well as better laser designs have renewed interest in these sources; their wavelengths are of considerable interest for a number of fluorescent applications ***(7)***. Integration of He–Ne 543- and 594-nm lasers onto a modern benchtop cuvette flow cell analyzer will therefore be described.

*Violet diode lasers (VLD).* The gallium nitride and indium gallium nitride UV and violet laser diodes recently developed by Nakamura and colleagues have been adapted to several commercial flow cytometers; they provide a valuable wavelength range (395–410 nm) for the excitation of phenotyping fluorochromes such as Cascade and Pacific Blue, and expressible fluorescent proteins such as cyan fluorescent protein ***(13–17)***. These laser continue to improve in power level and beam profile; several such sources will be reviewed here, both on cuvette and jet-in-air flow cell systems.

*Near-UV diode lasers (NUVLD).* Indium gallium nitride laser diodes have been developed that can extend into the near-UV range (370–385 nm). At this writing, these lasers are beginning to achieve useful power levels with adequate laser lifetimes (>5000 h). While these lasers are already proving useful for the determination of cell cycle using UV-excited DNA probes, they may soon be applicable to other UV-based applications such as calcium flux detection with the fluorogenic chelator indo-1 (an application requiring a shorter wavelength than that provided by violet sources). A prototype source has been installed onto a cuvet system and will be reviewed here.

Although these lasers are only a sampling of novel small sources available for flow cytometry, they provide general principles for integrating these devices into flow cytometers. These excitation sources are rapidly becoming the dominant source of laser light for flow cytometry, a technological trend that should continue.

## 2. Materials

1. Lasers: The lasers and their manufacturers are listed below. Although this list should not be considered exhaustive or exclusive, some products are manufacturer specific, or conform to custom requirements adhered to by particular suppliers. This will be noted where it occurs.
   a. He–Ne green 543-nm laser: The laser used in this study was manufactured by Research Electro-Optics, Inc. (Boulder, CO) and emitted at 3 mW following warmup. No power stabilization circuitry was incorporated into this system. Beam was circular to within 1.1 mradians, a beam diameter of 1.2 mm at the outermost beam fringe (**Fig. 1**). This laser showed true $TEM_{00}$ beam mode and an excellent Gaussian beam profile (*see* **Note 1**). Other manufacturers include Melles Griot (Boulder, CO) and JDS Uniphase (San Jose, CA).

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Fig. 1. *(see facing page top)* **(A)** He–Ne green 543-nm laser. **(B)** Beam profile of the above laser with no beam expansion, or **(C)** with beam expansion using a 5X adjustable optic.

Fig. 2. *(see facing page bottom)* **(A)** He–Ne yellow 594-nm laser. **(B)** Beam profile of a Melles Griot standard laser (2.7 mW. **(C)** Beam profile of a Melles Griot bottle-spec'd laser (4.0 mW). **(D)** Beam profile of a Research Electro-Optics laser (7.0 mW). All profiles taken with no beam expansion.

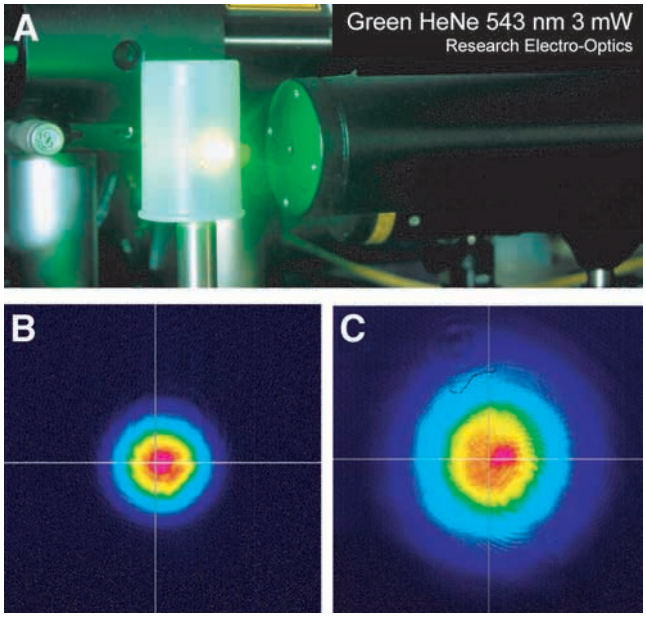

A
Green HeNe 543 nm 3 mW
Research Electro-Optics
B
C

Fig. 1.

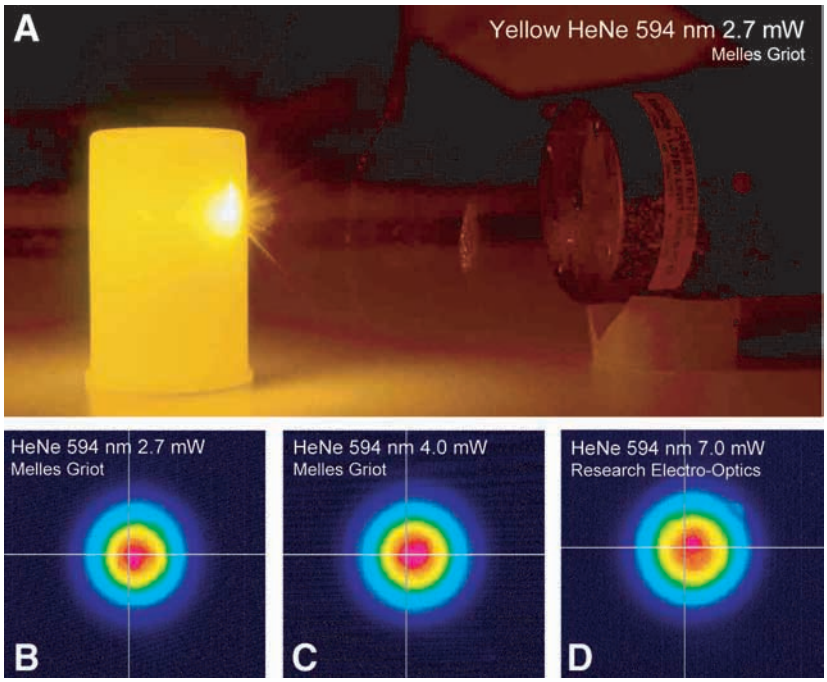
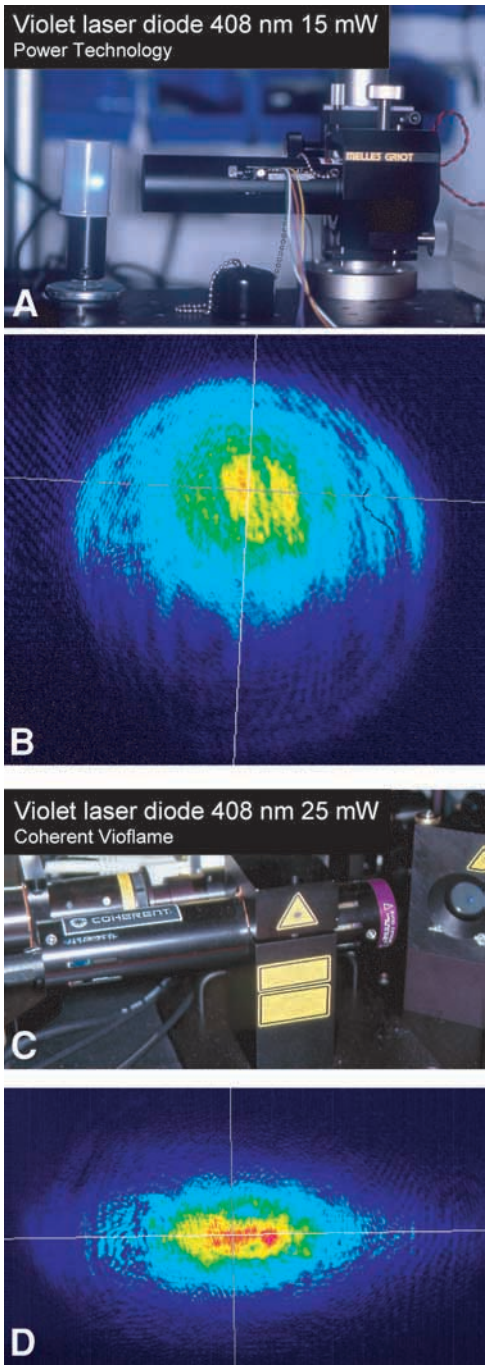

A
Yellow HeNe 594 nm 2.7 mW
Melles Griot
HeNe 594 nm 2.7 mW
Melles Griot
B
HeNe 594 nm 4.0 mW
Melles Griot
C
HeNe 594 nm 7.0 mW
Research Electro-Optics
D

Fig. 2.

b. He–Ne yellow 594-nm laser: Three yellow He–Ne lasers were evaluated in this study; a stock laser from Melles Griot emitting at 2.7 mW, a custom laser from Melles Griot using the plasma tube with the highest power rating from an entire manufacturing lot, emitting at 4 mW, and a custom laser from Research Electro-Optics with an extended coaxial plasma tube, emitting at 7 mW (**Fig. 2**). These lasers also showed true $TEM_{00}$ beam modes and excellent Gaussian beam profiles.
c. Violet diode 408 nm (Power Technology): This laser was manufactured by Power Technology, Inc. (Alexander, AR) and emitted at 15 mW following warmup. Violet laser diodes are inherently multimodal and require dual prisms to circularize the beam. The beam dimensions following circularization was approx 3 mm × 2.5 mm at the outermost beam fringe, with a somewhat asymmetrical single-mode beam (*see* **Fig. 3B** and **Note 2**).
d. Violet diode 408 nm (Coherent): This laser was manufactured by Coherent, Inc. (Santa Clara, CA) under the tradename Vioflame, and emitted at 25 mW following warmup. The beam dimensions following circularization were elliptical and approx 3.5 mm × 1 mm at the outermost beam fringe, with a reasonably symmetrical single-mode beam (**Fig. 3D**).
e. Near-UV diode 372 nm: This laser was also manufactured by Power Technology, Inc. and emitted at approx 1.5 mW following warmup. The beam dimensions following circularization were roughly elliptical and approx 4 mm × 1.5 mm at the outermost beam fringe, with a reasonably symmetrical single-mode beam.
f. Traditional gas laser sources: Where possible small lasers are compared to traditional gas lasers sources. The green He–Ne and violet diode sources were compared to the green (530 nm) and violet (407 nm) lines of a Coherent I-302C water-cooled krypton-ion laser.

2. Power meter: Laser power was estimated using a NIST-traceable 2W broadband power meter (400 nm–2 mm) with a thermopile graphite detector head from Melles Griot. Comparable power meters are available from many manufacturers, and should be selected based on the power level of the lasers to be tested.
3. Charge-coupled device (CCD) beam profiling system: CCD chip-based beam profiling systems are useful for determining modality, beam circularity and shape, and Gaussian (or non-Gaussian) beam distributions. This study used the WinCamD system manufactured by DataRay, Inc. (Boulder Creek, CA) and utilizes a 6.3 mm × 4.7 mm Sony CCD chip with neutral density filter blockers. Care should be taken when using CCD chip-based beam profiling optics to attenuate the laser beam and prevent damage to the CCD element (*see* **Note 3**).
4. Flow cytometers:

Fig. 3. *(see facing page)* **(A)** Power Technology 408-nm violet laser diode at 15 mW. **(B)** Beam profile of this laser. **(C)** Coherent Vioflame 408-nm violet laser diode at 25 mW. **(D)** Beam profile of this laser.


Violet laser diode 408 nm 15 mW
Power Technology
A
B
Violet laser diode 408 nm 25 mW
Coherent Vioflame
C
D

a. FACSVantage DiVa Cell Sorter (BD Biosciences, San Jose, CA): This instrument is a typical jet-in-air or stream-in-air cell sorter, where the laser beam directly intercepts the cell stream with no intervening cuvet or flow cell. The instrument uses a set of steering dichroics and prisms to aim the lasers, a flat prism to shape the beams, a standard 50-mm focal length laser focusing lens to focus them onto the sample stream, and a 70-µm sample/sheath nozzle. Data acquisition and analysis was carried out in digital mode. Long- or shortpass dichroics and narrow bandpass filters are used to separate and isolate fluorescent signals.
b. LSR II (BD Biosciences): This instrument utilizes a quartz cuvet or flow cell rather than a jet-in-air system. As with the FACSVantage DiVa, a set of reflecting dichroics are used to steer the beams to the cuvet, with an intersecting flat prism and a laser focusing lens for beam shaping and focusing. In contrast to the FACSVantage DiVa, the resulting fluorescent signals are focused onto a set of pinholes coupled to fiber optics, which direct the signals to the appropriate banks of PMT detectors.

5. Alignment standards: Fluorochrome-tagged microspheres are used as quality control and alignment standards for flow cytometers; microspheres with graded levels of incorporated fluorochrome can also be used to gauge the sensitivity and signal-to-noise ratios of detectors. The following bead standards were used for laser evaluation, although many alternatives are available:
   a. He–Ne green 543-nm: Green lasers provide excellent excitation of phycoerythrin (PE) and other yellow- and orange-emitting fluorochromes. The PE-like bead standard is therefore useful for this laser line. The Linear Flow Carmine bead array (Molecular Probes, Eugene, OR) was used for this source; this bead series has an incorporated proprietary fluorochrome at levels of 100%, 10%, 2%, 0.4%, 0.1%, and 0.02%, with the highest level being an arbitrary value. An example of these beads with He–Ne 543-nm excitation is shown in **Fig. 4A**.
   b. He–Ne yellow 594 nm: Yellow lasers can excite Texas Red and allophycocyanin, which emit in the orange to red range. The InSpeck Deep Red bead array (Molecular Probes) was used for this source; this bead series has an incorporated red-emitting fluorochrome at levels of 100%, 30%, 10%, 3%, 1%, and 0.3% and unlabeled, with the highest level being an arbitrary value. An example of these beads with He–Ne 594 nm excitation is shown in **Fig. 5A**.
   c. Violet diode 408 nm and near-UV diode 372 nm: Violet lasers excite fluorochrome such as Cascade Blue and Pacific Blue. Although microbead standards specific for violet excitation are not commonly available, UV-excited bead standards are useful for this purpose. The InSpeck Blue bead array (Molecular Probes) was used for this source; this bead series has an incorporated blue-emitting fluorochrome at levels of 100%, 30%, 10%, 3%, 1%, and 0.3% and unlabeled, with the highest level being an arbitrary value. For laser alignment, yellow-green 2-µm beads from Polysciences (Warrington, PA) were used along with a fluorescein isiothiocyanate (FITC) excitation filter; while these beads are mainly used to align instruments with blue-green 488-nm lasers, they are also well-excited by UV and violet sources.
6. Cells and fluorochromes: Cells were labeled with fluorochromes appropriate to

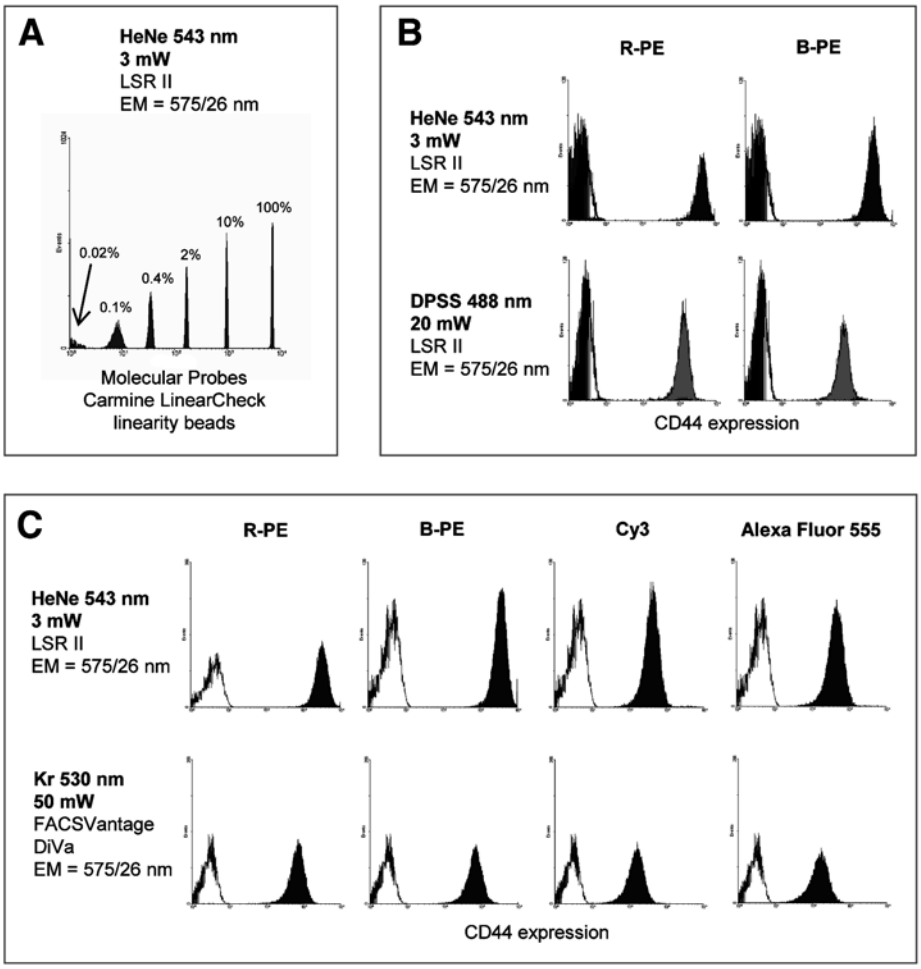
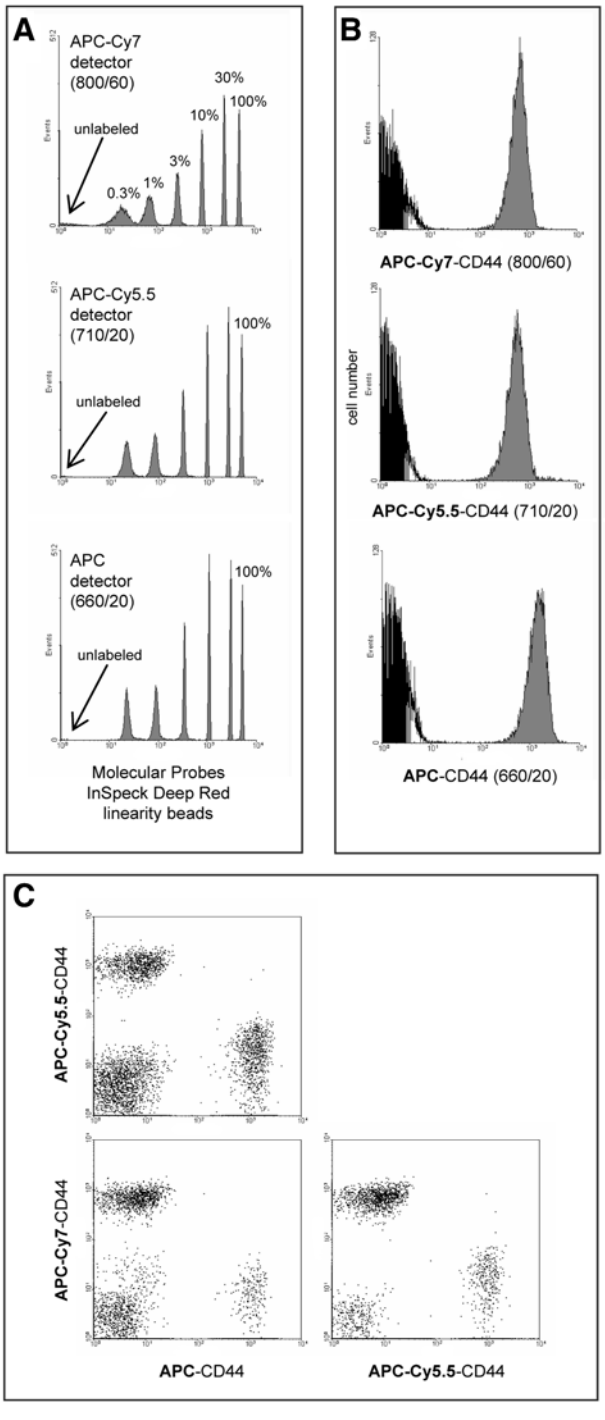



Fig. 4. **(A)** Analysis of Molecular Probes LinearCheck Carmine microspheres using He–Ne 543 nm excitation on the LSR II. Percentage relative fluorescence of each bead population is shown. **(B)** Analysis of EL4 thymoma cells labeled with biotin-anti-CD44 followed by streptavidin conjugated to R-PE **(left column)** or B-PE **(right column)**, using either He–Ne 543 nm **(top row)** or DPSS 488-nm excitation **(bottom row)**. **(C)** Analysis of EL4 cells labeled as above for **(left to right columns)** R-PE, B-PE, Cy3, or Alexa Fluor 555, using either He–Ne 543 nm **(top row)** or krypton-ion 530 nm **(bottom row)** excitation. Emission filter used for all sample analyses is indicated (EM = 575/26 nm).

the evaluated lasers. For most evaluations, EL4 mouse thymoma cells (ATCC, Manassas, VA) were labeled with biotin-conjugated anti-CD44 or CD90 followed by a streptavidin conjugate of the fluorochrome of interest. For DNA content analysis using violet or near-UV diode lasers, chicken red blood cells (CRBCs),


A
APC-Cy7 detector (800/60)
unlabeled
0.3%
1%
3%
10%
30%
100%
APC-Cy5.5 detector (710/20)
unlabeled
100%
APC detector (660/20)
unlabeled
100%
Molecular Probes InSpeck Deep Red linearity beads
B
APC-Cy7-CD44 (800/60)
cell number
APC-Cy5.5-CD44 (710/20)
APC-CD44 (660/20)
C
APC-Cy5.5-CD44
APC-Cy7-CD44
APC-CD44
APC-Cy5.5-CD44

trout red blood cells (TRBCs) or calf thymus nuclei (CTNs) were labeled with the UV-excited DNA probe DAPI at 2 µg/mL prior to analysis. These cell standards can be used for evaluating any new laser configuration.

a. He–Ne green 543 nm: The green He–Ne was used to excite EL4 cells labeled with both the R- and B- form of PE. R-PE is the most commonly used form of this phycobiliprotein; however, B-PE has a somewhat longer wavelength excitation/emission spectrum and can be useful for green excitation sources. The ability of the green He–Ne laser to excite low molecular weight fluorochromes including Cy3 and Alexa Fluor 555 was also assessed. Both R-PE- and B-PE-conjugated strepavidin were obtained from ProZyme, Inc. (San Leandro, CA). Cy3 was obtained from Jackson ImmunoResearch (West Grove, PA), and Alexa Fluor 555 from Molecular Probes.
b. He–Ne yellow 594 nm: Yellow He–Ne lasers can excite allophycocyanin (APC) and its tandems with efficiency approaching red He–Ne lasers; additionally, they can also excite Texas Red, allowing simultaneous analysis of this useful fluorochrome, APC, and its tandems. EL4 cells, EL4 cells labeled with APC, the APC tandem probes APC-Cy5.5 and APC-Cy7, and Texas Red (and its Molecular Probes equivalent Alexa Fluor 594) were therefore all tested. APC, APC-Cy5.5, and APC-Cy7 conjugated streptavidin were obtained from Caltag (Burlingame, CA). Texas Red and Alexa Fluor 594 conjugated streptavidin were obtained from Molecular Probes.
c. Violet diode 408 nm: Violet diode lasers can excite the phenotyping fluorochromes Cascade Blue and Pacific Blue, as well as cyan fluorescent protein (CFP). EL4 cells labeled with Cascade Blue and Pacific Blue were therefore used as a cell standard. Cascade Blue and Pacific Blue conjugated streptavidin were obtained from Molecular Probes.
d. Near-UV diode 372 nm: The relatively low power output of this prototype laser (372 nm) made it unsuitable for exciting UV phenotyping dyes such as 7-aminomethylcoumarin (AMCA) or Alexa Fluor 350. However, the DNA standards (CRBCs, TRBCs, and CTNs) were used to evaluate this source for

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Fig. 5. *(see opposite page)* **(A)** Analysis of Molecular Probes InSpeck Deep Red microspheres through detectors equipped with filters for APC-Cy7 (800/60-nm filter), APC-Cy5.5 (710/20 nm), and APC (660/20 nm) using 594-nm He–Ne excitation on the LSR II. A 735-longpass dichroic was used to split the APC-Cy7 signal from APC-Cy5.5 and APC, and a 695-longpass to split the APC-Cy5.5 signal from APC. Percentage relative fluorescence of each bead population is shown. **(B)** Analysis of EL4 thymoma cells labeled with biotin-anti-CD44 followed by streptavidin conjugated to APC-Cy7 **(top)**, APC-Cy5.5 **(middle)**, or APC **(bottom)** using the above filter/dichroic configuration. **(c)** Simultaneous analysis of the above three fluorochromes using a "cocktail" of APC-Cy7, APC-Cy5.5, and APC conjugated EL4 cells using the above filter/dichroic configuration. Emission filters used for all sample analyses are indicated in parentheses.

DNA content measurement using the DNA probe DAPI.

## 3. Methods

Laser safety: All of the lasers described below are class IIIa or IIIb laser sources and should be handled with caution (*see* **Note 4**).

### 3.1. He–Ne Green 543-nm Laser

1. Laser and instrument configuration: The He–Ne 543-nm laser is shown in **Fig. 1A**. The beam profile shows an excellent Gaussian distribution (**Fig. 1B**). This laser was mounted on the BD LSR II in place of the conventional He–Ne 633-nm laser. The cuvet and pinhole design of the LSR II light collection optics frequently requires some laser beam shaping, expanding the brightest point of the beam somewhat; this is accomplished using a 5X beam expanding optic (Newport Corporation, Irvine, CA), resulting in a wider beam profile and flatter beam top (*see* **Fig. 1C** and **Note 1**).
2. Alignment and sensitivity: LinearCheck Carmine alignment microspheres were analyzed on the green He–Ne-equipped LSR II both for alignment and assessment of sensitivity. Alignment was carried out by a combination of adjusting the laser dichroic mirrors and the beam expanding optic. **Figure 4A** shows the results from the microsphere analysis—excellent peak CVs are achieved, and the dim 0.1% bead population can be easily distinguished from the lowest 0.02% component.
3. Fluorochrome analysis: EL4 cells labeled with either R-PE or B-PE were analyzed with the He–Ne 543-nm laser and compared to excitation with a DPSS 488-nm 20-mW laser source on the same instrument (**Fig. 4B**). Blue-green 488-nm laser light is most commonly used to excite PE to its ubiquity on most bench-top sorters. However, the He–Ne 543 nm gave much better excitation than the 488-nm line, despite a far lower power level. The ability of the He–Ne 543 nm to excite cells at more than adequate levels is further illustrated in **Fig. 4C**, where green He–Ne excitation of R-PE, B-PE, and the low molecular weight fluorochromes Cy3 and Alexa Fluor 555 was compared to green excitation with a krypton-ion 530-m laser at 50 mW on the FACSVantage DiVa. The more efficient light collection optics on the cuvet instrument combined with green He–Ne excitation gave better sensitivity than a more powerful gas laser on a jet-in-air instrument.

### 3.2. He–Ne Yellow 594-nm Laser

1. Laser and instrument configuration: A typical He–Ne 594-nm laser is shown in **Fig. 2A**. Three He–Ne yellow 594-nm lasers were evaluated, with the objective of obtaining a laser with maximum power output (a problem with yellow He–Ne lasers, as the emission of the yellow line is relatively inefficient). Beam profiles for lasers emitting at 2.7, 4, and 7 mW are shown in **Fig. 2B,C,D**. As with the green He–Ne, excellent Gaussian profiles were obtained. Beam expansion to maximize the brightest point of the beam was also useful for these sources (*see* **Note 1**). The lasers were individually mounted on the LSR II in the default red

He–Ne position as was done for the green.

2. Alignment and sensitivity: The LSR II was equipped to detect three fluorochromes with the yellow He–Ne; therefore, the detectors were configured to detect APC (with a 660/20 nm narrow bandpass filter), APC-Cy5.5 (710/20 nm), and APC-Cy7 (800/60 nm). Analysis of the InSpeck Deep Red bead array gave excellent sensitivity in all detectors, with the dimmest 0.3% microsphere population easily distinguishable from the unlabeled spheres (**Fig. 5A**).
3. Analysis of APC and APC tandem conjugates: EL4 cells labeled with APC, APC-Cy5.5, or APC-Cy7 were all well excited with the yellow laser (**Fig. 5B**). Comparative studies between yellow and red He–Ne lasers show a negligible loss of APC excitation with the yellow sources; these lasers are quite adequate for use with red-excited phycobiliproteins (data not shown). Three-color analysis of a "cocktail" of APC-, APC-Cy5.5-, and APC-Cy7-labeled EL4 cells showed that all three fluorochromes could be simultaneously analyzed with reasonable color compensation values, as is commonly done with red laser sources.
4. Analysis of Texas Red: The most significant advantage of replacing a red He–Ne laser with a yellow is the ability to analyze Texas Red in addition to APC and its tandems. Texas Red and its more recent derivatives (such as Alexa Fluor 594) are very bright fluorochromes; however, they now see little use in flow cytometry, primarily because of the lack of simple yellow excitation sources. Dye head lasers emitting in the 580- to 620-nm range have been typically required to excite Texas Red; these lasers require a powerful water-cooled gas laser as a dye "pump," and are very maintenance-intensive. Yellow He–Ne lasers can excite Texas Red extremely well, allowing it to be combined with APC and its tandems for up to four-color analysis off one yellow source. Yellow He–Ne excitation of Texas Red and Alexa Fluor 594 (using a 630/22 nm filter) is shown in **Fig. 6A**, and in combination with APC and APC-Cy7 in **Fig. 6B**. Yellow He–Ne excitation therefore allows the addition of an additional fluor to APC and its tandems.

### *3.3. Violet Laser Diode 408 nm*

1. Laser and instrument configuration: Two typical violet laser diodes are shown in **Fig. 3**. A Power Technology VLD is shown in **Fig. 3A**, with its beam profile in **Fig. 3B**. A Coherent Vioflame VLD and its beam profile are shown in **Fig. 3C,D**. Unlike He–Ne lasers, diode lasers often have multimodal and highly asymmetrical beam configurations; commercial lasers usually attempt to correct this with a pair of cylindrical lenses mounted at 90° angles to one another, circularizing the beams to some degree (*see* **Notes 1** and **2**). The Power Technology beam has been circularized but with an uneven distribution over the entire beam diameter; however, the brightest point of the beam has been roughly centered (**Fig. 3B**). The Coherent beam has a more uniform elliptical shape (**Fig. 3D**). In both cases, the lasers were mounted on either the LSR II or FACSVantage DiVa flow cytometers and rotated until the broadest beam axis was horizontal; this resulted in a more elliptical beam profile on the flow cell or sample stream. This profile is desirable

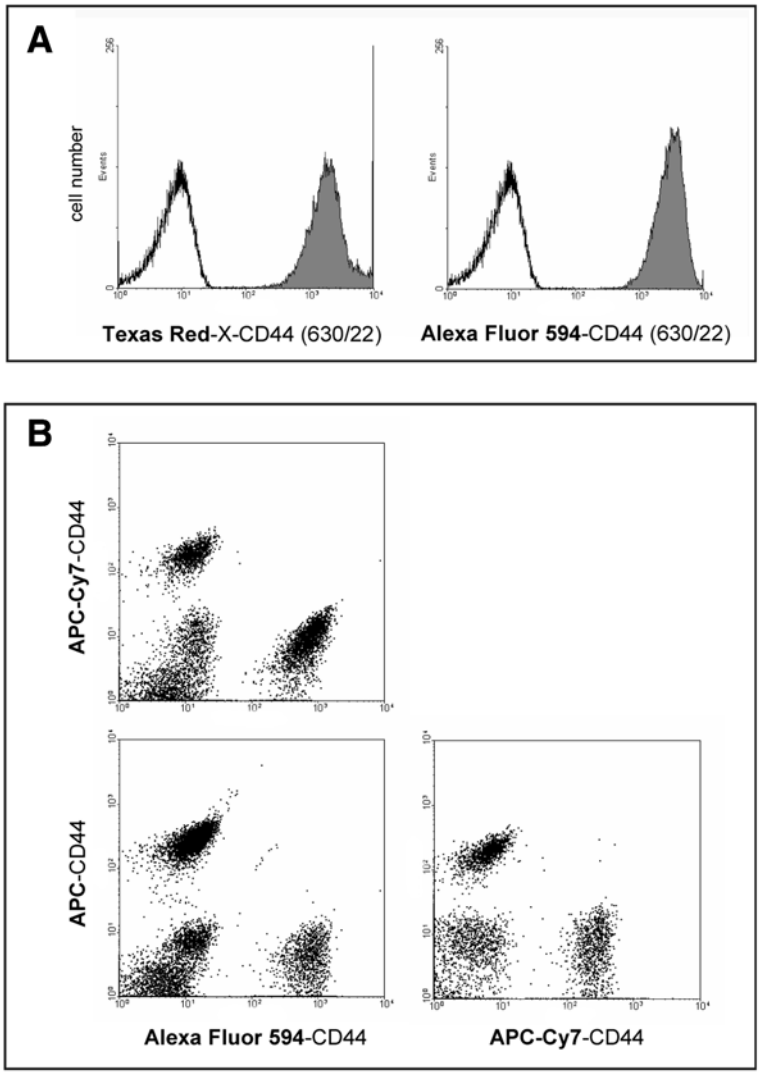



Fig. 6. **(A)** Analysis of EL4 cells labeled with biotin-anti-CD44 followed by streptavidin conjugated to Texas Red **(left)** or Alexa Fluor 594 **(right)** with He–Ne 594-nm excitation on the LSR II. **(B)** Simultaneous analysis of the above fluorochromes using a "cocktail" of APC-Cy7-, APC-, and Alexa Fluor 594-conjugated EL4 cells using the above filter/dichroic configuration. Emission filters used for all sample analyses are indicated in parentheses.

for good cell illumination, since the cell path will be maximally illuminated horizontally but will have a reduced vertical cross-section.
The relatively large power outputs of these lasers make them applicable not only for cuvet instruments such as the LSR II, but also for jet-in-air instruments the FACSVantage DiVa cell sorter. These lasers were therefore mounted on both instruments. The mounting system for the cell sorter is shown in **Fig. 7**. The mounting hardware (identified in **Fig. 7A,B**) was obtained from Newport Instruments (Irvine, CA), although any optical component supplier can provide these standard parts. This mounting system allowed both translational and rotational movement along all the relevant axes. The lasers are shown mounted on the cell sorter in **Fig. 7C,7D**.

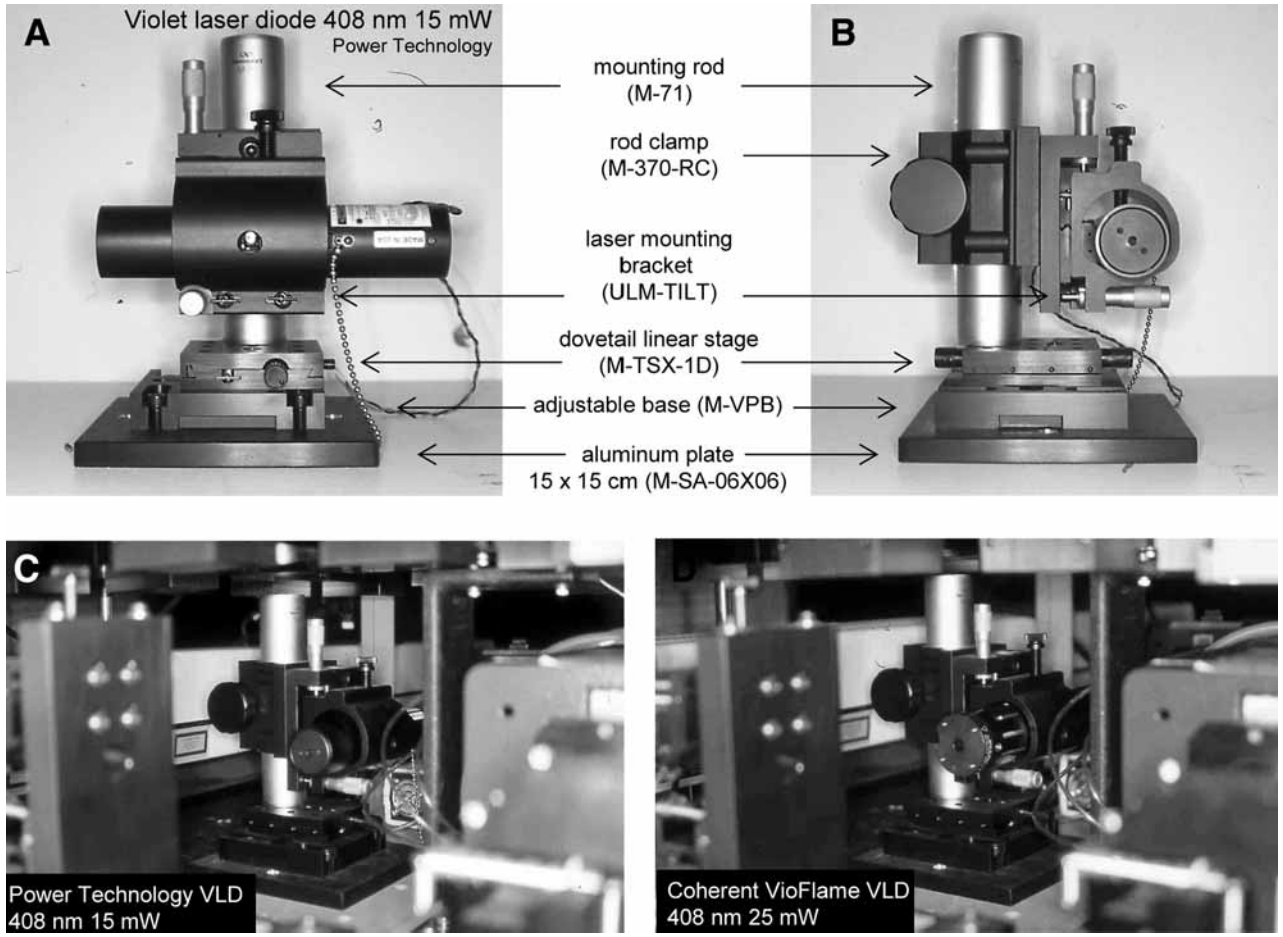
2. Alignment and sensitivity: All alignments were carried out using the InSpeck Blue microspheres.
3. Fluorochrome analysis: Violet laser diodes excite Cascade Blue and Pacific Blue particularly well. This is shown in **Fig. 8A**, where Cascade Blue and Pacific Blue labeled EL4 cells were analyzed on the LSR II. Interestingly, mounting the same lasers on the FACSVantage DiVa jet-in-air sorter give similar signal-to-noise ratios to that seen on the LSR II for both lasers (**Fig. 8B**). Despite their differing beam profiles and power levels, both lasers in this study performed comparably. This suggests that, while these lasers work well on cuvet instruments, their power levels make them applicable to open-stream systems as well despite their reduced light collecting capabilities. In fact, VLDs provide similar excitation efficiency to more powerful gas lasers emitting in the violet; the comparison in **Fig. 8C** shows that the 15-mW VLD excited Cascade and Pacific Blue labeled cells as well as a krypton-ion source emitting 50 mW at 407 nm.

### *3.4. 372-nm Near-UV Laser Diode*

1. Laser and instrument configuration: An engineering prototype 372-nm diode is shown in **Fig. 9A**, and mounted on the LSR II in **Fig. 9B**. Despite its low power, the laser gave excellent results for cell cycle analysis; analysis of CRBC, TRBC, and CTN standards labeled with the DNA binding dye DAPI at 2 μg/mL gave excellent CVs (**Fig. 9C**). Violet laser diodes can also be used to measure DNA content in 4′,6′-diamidino-2-phenylincole (DAPI)-labeled cells; however, the UV wavelength gave better resolution despite a lower power level (**Fig. 9C**). When these lasers achieve higher power levels and diode lifetimes, they may prove very useful not only for cell cycle analysis, but dimmer UV-based applications such as indo-1 calcium measurement and Hoechst 33342 "side population" stem cell analysis as well.

### *3.5. Conclusions*

This chapter has shown practical examples of small, low-power laser integration into flow cytometers. Results obtained with these sources can be comparable to those obtained using more traditional lasers sources. Incorporation of


A
Violet laser diode 408 nm 15 mW
Power Technology
B
mounting rod
(M-71)
rod clamp
(M-370-RC)
laser mounting
bracket
(ULM-TILT)
dovetail linear stage
(M-TSX-1D)
adjustable base (M-VPB)
aluminum plate
15 x 15 cm (M-SA-06X06)
C
Power Technology VLD
408 nm 15 mW
D
Coherent VioFlame VLD
408 nm 25 mW

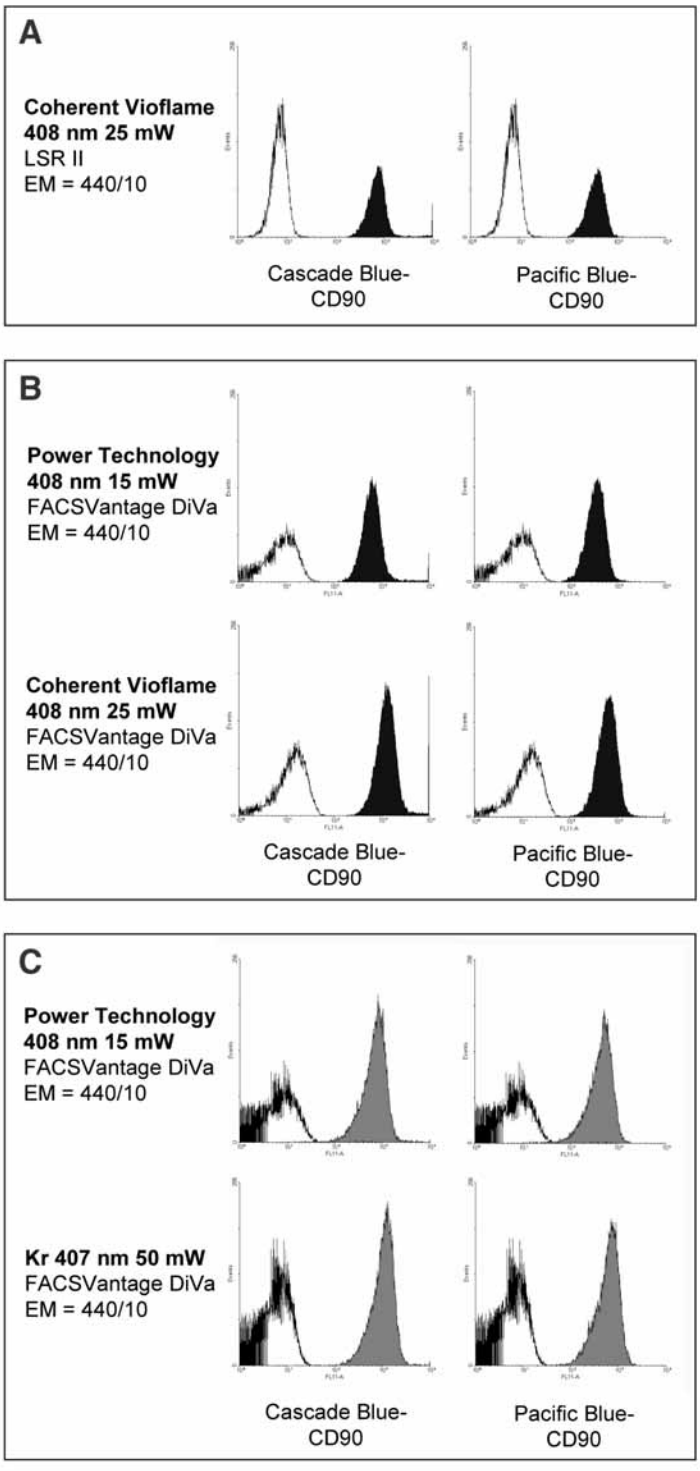
these lasers into commercial instruments is already well under way; it is expected that this technology will become ubiquitous in modern instrument design.

## 4. Notes

1. Beam profile and mode: Special attention should be paid to beam profile when integrating a new laser into a flow cytometric system. Gas lasers (both large and small) traditionally produce a $TEM_{00}$ or single-mode profile, appearing as a symmetrical Gaussian distribution. These beams are often subsequently shaped to produce an elliptical spot that will illuminate the entire sample stream in the horizontal axis, with a flatter beam top. Small gas lasers (such as He–Ne) usually produce extremely uniform Gaussian beams. Diode lasers, on the other hand, frequently produce multimodal (or mixed mode) beams with multiple peaks and valleys; these can be shaped with cylindrical optics to produce a roughly circular or elliptical spot. When adapting one of these lasers to a flow cytometer, the beam profile should be measured at the distance the beam will travel to the cell intercept point, to ensure that the shape is appropriate or at least acceptable for stream interrogation. In the case of He–Ne lasers, beam shape can be modified with beam expanding optic. For diode lasers, most commercial models allow some adjustment to the cylindrical lenses, with the help of the manufacturer.
2. Circularization optics: Modifying the beam shape in diode lasers with prisms or cylindrical optics inevitably reduces the power output, sometimes significantly. This decrease can run up to 30% in some cases, and should be taken into account when choosing diode lasers.
3. CCD camera profiling systems: CCD chip beam profiling systems can suffer permanent damage if too powerful a laser beam is directed against their surface. The manufacturers usually provide the necessary equations to calculate the maximum beam power their systems can endure; these should be followed closely. Neutral density filters can usually be inserted in the beam path to attenuate more powerful lasers and still allow their beam profile to be measured.
4. Safety: Although these lasers described below have power outputs well below that typically observed from water-cooled gas sources, eye damage can still result from direct beam exposure. The violet and near-UV sources in particular can cause both eye and skin damage. Proper protective eyewear and beam shielding is highly recommended when working with any class III laser source.

## Acknowledgments

Fig. 7. *(see opposite page)* **(A,B)** VLD mounting system for integration into the FACSVantage DiVa cell sorter (*front and side views*). Part descriptions and numbers correspond to materials available from Newport Corporation. **(C)** Power Technology VLD mounted on the cell sorter. **(D)** Coherent Vioflame VLD mounted on the cell sorter.


A
Coherent Vioflame
408 nm 25 mW
LSR II
EM = 440/10
Cascade Blue-
CD90
Pacific Blue-
CD90
B
Power Technology
408 nm 15 mW
FACSVantage DiVa
EM = 440/10
Coherent Vioflame
408 nm 25 mW
FACSVantage DiVa
EM = 440/10
Cascade Blue-
CD90
Pacific Blue-
CD90
C
Power Technology
408 nm 15 mW
FACSVantage DiVa
EM = 440/10
Kr 407 nm 50 mW
FACSVantage DiVa
EM = 440/10
Cascade Blue-
CD90
Pacific Blue-
CD90

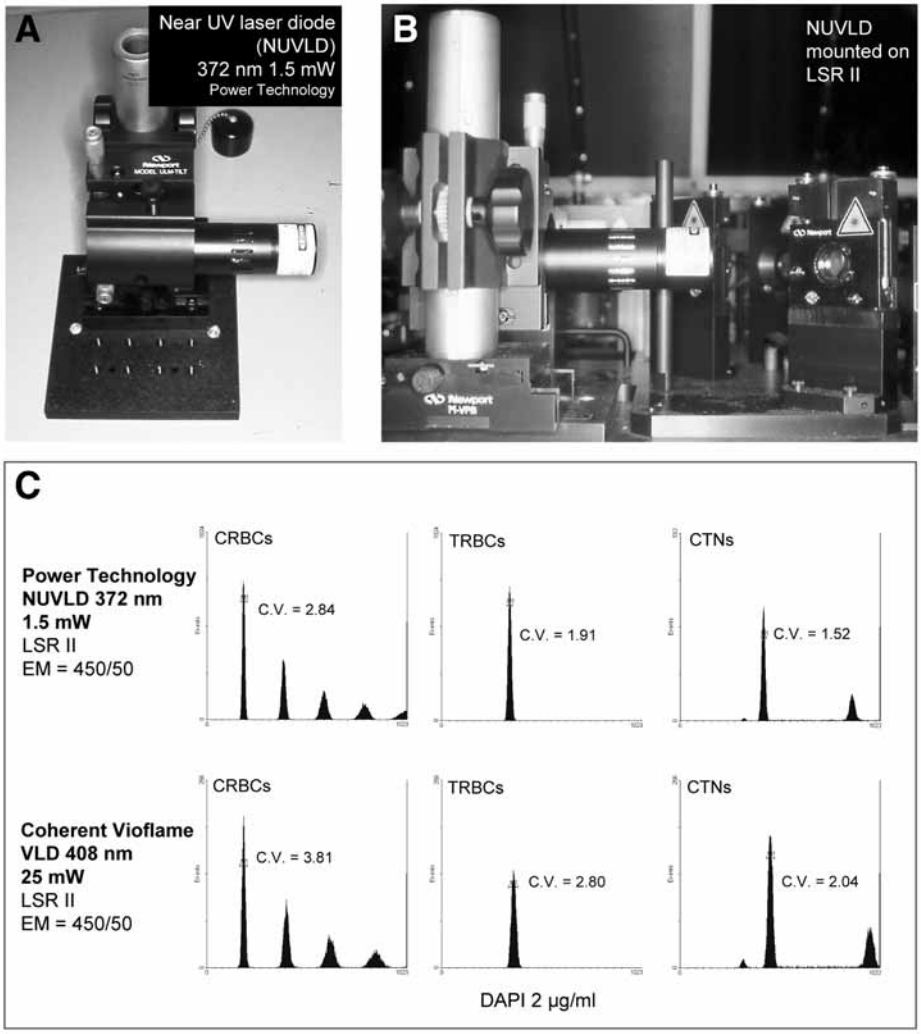



Fig. 9. **(A)** Power Technology near-UV laser diode 372 nm at 1.5 mW. **(B)** NUVLD mounted on the LSR II. **(C)** DNA content analysis of chicken red blood cells (CRBCs, **left column**), trout red blood cells (TRBCs, **middle column**), and calf thymus nuclei (CTNs, **right column**) labeled with DAPI at 2 µg/mL using the NUVLD **(top row)** or VLD **(bottom row)** on the LSR II. Singlet peak CVs are indicated. Emission filters used for all sample analyses are indicated (EM).

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Fig. 8. *(see opposite page)* **(A)** Analysis of EL4 cells labeled with biotin-anti-CD90 followed by streptavidin conjugated to Cascade Blue **(left)** or Pacific Blue **(right)** using VLD excitation on the LSR II. **(B)** Analysis of the above cells using either Power Technology or Coherent VLD excitation on the FACSVantage DiVa cell sorter. **(C)** Analysis of the above cells using either VLD or krypton-ion 407-nm excitation on the FACSVantage DiVa cell sorter. Emission filters used for all sample analyses are indicated (EM).

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