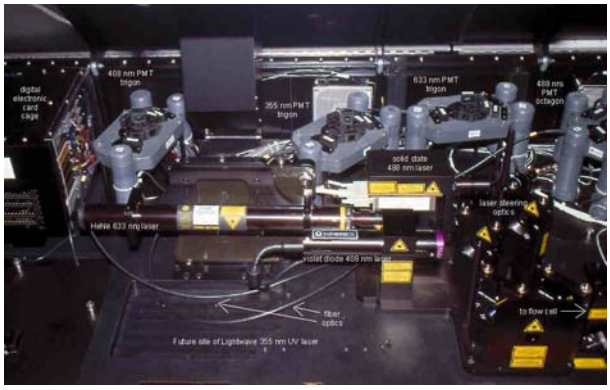
BD Biosciences LSR II.

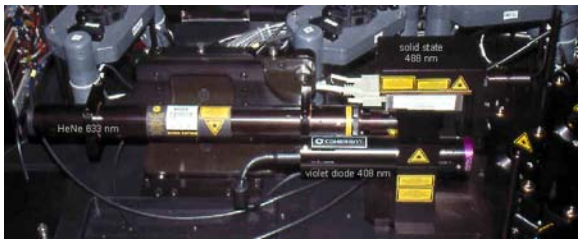
Our core recently acquired a LSR II, the latest benchtop flow cytometer developed by BD Biosciences and a significant redesign over the original LSR. This instrument is equipped with four laser sources: a Coherent Sapphire 20 mW 488 nm solid state laser (replacing the originally utilized air-cooled argon-ion laser), a JDS Uniphase helium-neon 17 mW 633 nm laser for red excitation, and a Coherent Vioflame 25 mW 408 nm violet laser diode. A Lightwave Electronics 355 nm frequency-tripled Nd-YAG laser will be added at the end of 2002 and will provide UV excitation, replacing the 325 nm HeCad laser used on earlier instruments. The instrument is currently set up to detect four fluorochromes using 488 nm excitation, two with the 633 nm HeNe, two with the 408 nm violet diode and two off the UV, giving us up to 10 fluorescent parameter detection simultaneously. The LSR II has full digital data acquisition/analysis capability using software similar to that used for the FACSVantage DiVa; unlike the DiVa, which still can acquire data under the old analog system, the LSR II is a fully digital system.

(Below). The LSR II. *Upper photo*, covers open; *bottom photo*, internal instrument configuration. The lasers are in the forward portion of the instrument; the flow cell, collection optics and fiber optic splitter are on the right forward portion of the instrument; the PMT "octagons" and "trigons" are located to the rear of the instrument.

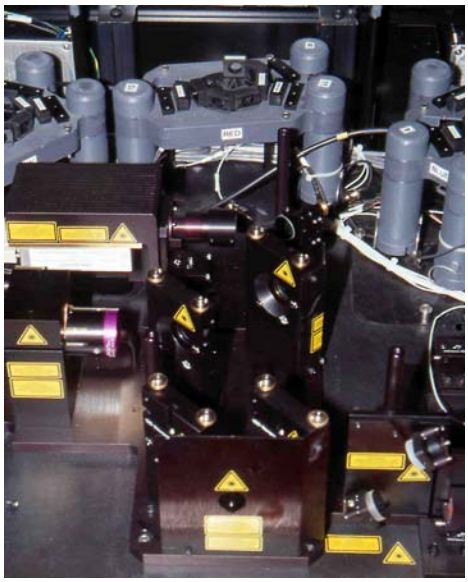


Lasers. The LSR II uses the same basic instrument "bench" as the old LSR. The lasers are mounted in roughly the same positions of the LSR, although the solid-state 488 nm is far smaller and has virtually no cooling requirements beyond a heat sink, saving considerable space. The beams are independently steered using Newport Instruments gimbaled optical mounts and laser dichroics accessible from above the beams paths, making multiple beam alignments far simpler than on the old LSR.

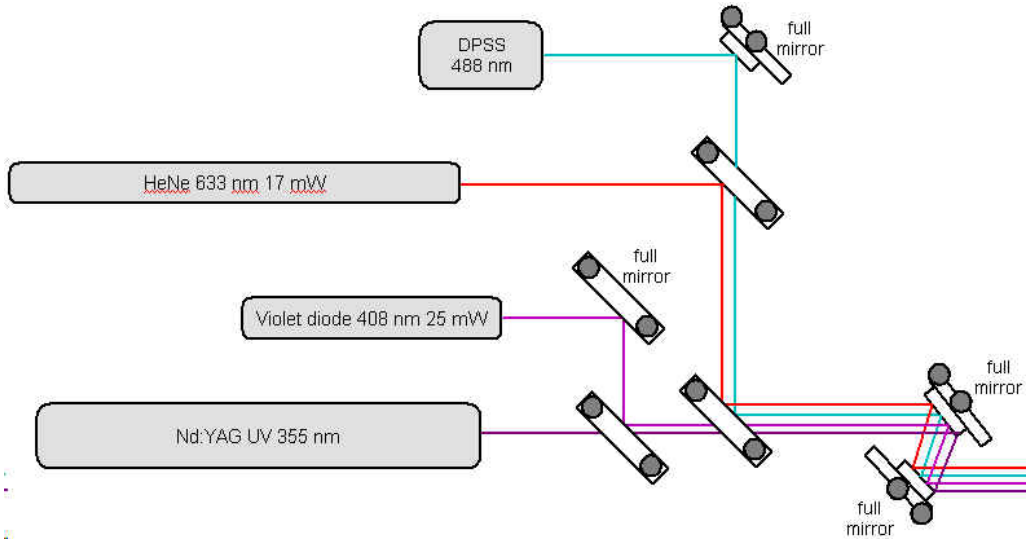
(Below). The LSR II lasers. From the back, the Coherent Sapphire 488 nm 20 mW solid state; the Cyonics 633 nm 20 mW HeNe gas laser; and the Coherent VioFlame 408 nm 15 mW violet laser diode. The Lightwave 355 nm UV laser will be added in the forward-most position later.



(Below). Laser beam steering optics.

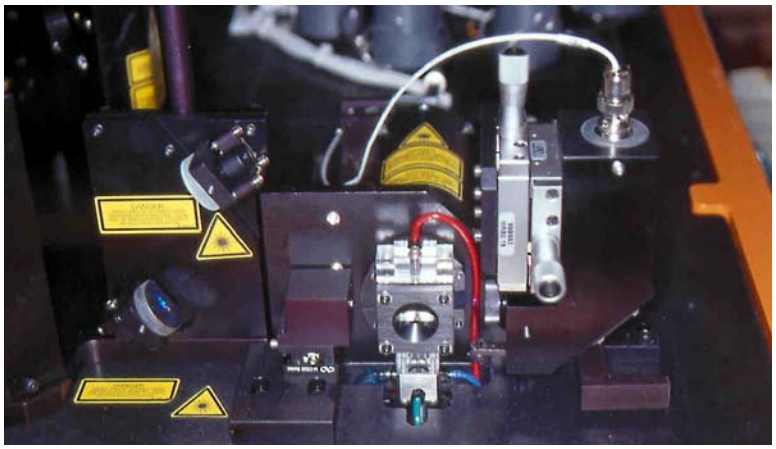


(Below). Schematic of laser beam steering optics. Beam order is arbitrary.



Collection optics and fiber optics. Like the LSR, the LSR II uses a standard FACSCalibur quartz flow cell. However, the LSR II utilizes an improved signal focusing objective, mated to the flow cell with an optical gel layer. This change in collection optics improves the numerical aperture of the system (BD claims) to 1.2, a major improvement over earlier systems. The resulting four sets of signals (each from one laser) are focused on four pinholes, behind which are four fiber optics light collection points. Each fiber optic channels signals to one of four PMT clusters. The pinhole system simplifies the laser delay settings, which remain relatively fixed after initial calibration and require no daily delay adjustment.

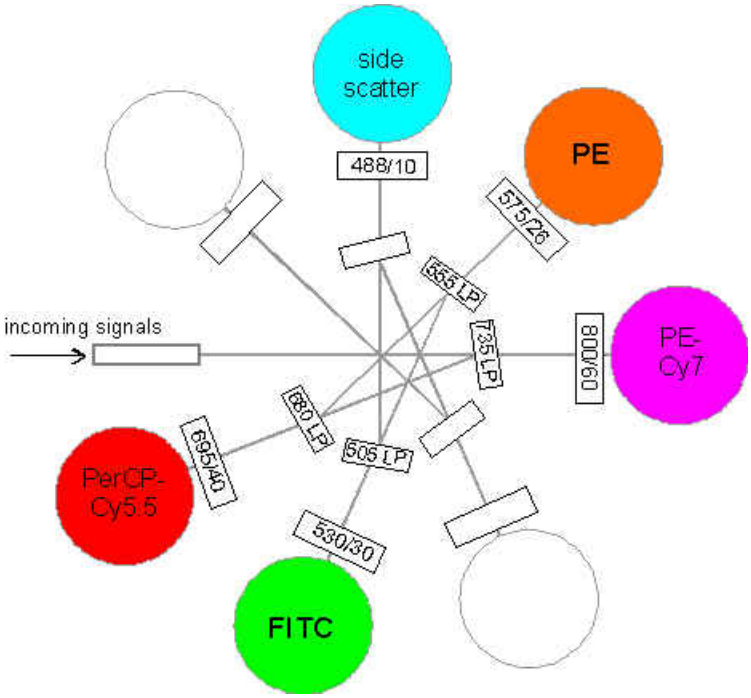
(Below). Flow cell, collection optics and fiber optic junction.



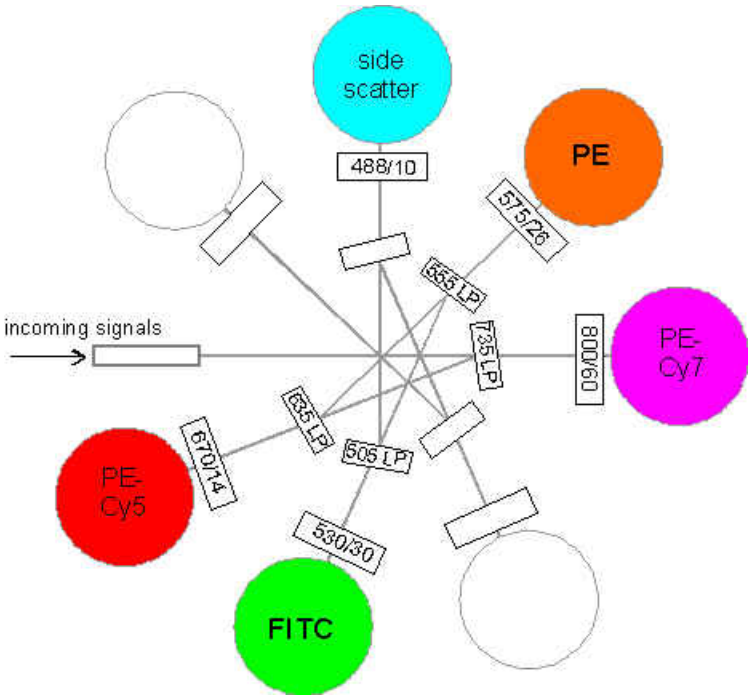
PMTs and filters/dichroics. The LSR II uses "clusters" of PMTs, dichroics and filters mounted in light-tight molded plastic units, referred to as "octagons" or "trigons" based on the number of detectors. The emission signals are transmitted into the PMT clusters via fiber optics, where the signals are appropriately passed or reflected into the multiple

detectors. Isolating each group of PMTs into a rigid, free-floating unit solves one of the problems with the old LSR, namely the mounting of the detectors directly on the optical bench, where they were very prone to mechanical disruption and loss of alignment. The instrument uses standard 25 mm narrow bandpass filters in removable mounts, allowing easy alteration of the optical configuration as well as the use of our existing filters. The longpass dichroics are a non-standard diameter and are spec'd to 13 degrees incidence of reflection to accomodate the design of the optical path; although we cannot use our own dichroics in this system, the existing ones are interchangeable.

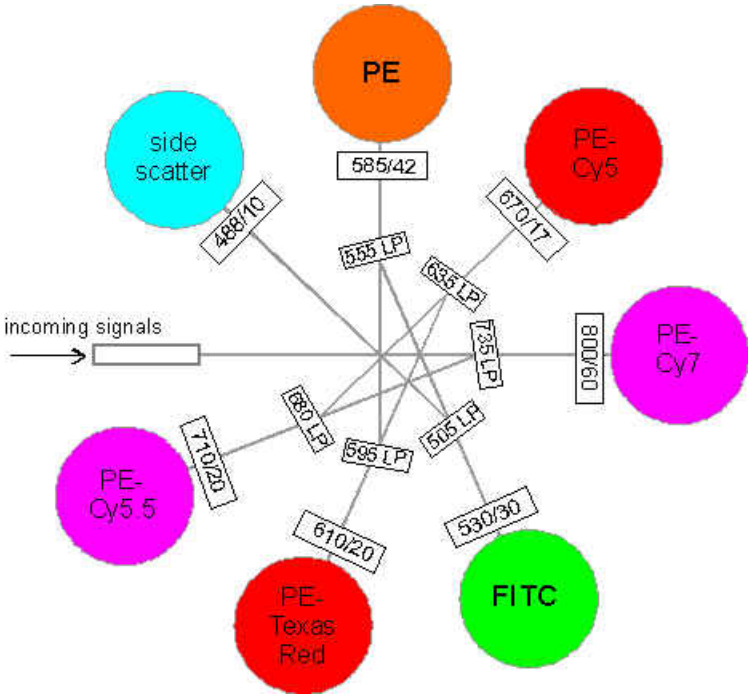
(Below). **Default configuration for the primary 488 nm detectors.** *Left*, the primary laser "octagon". *Right*, the current default configuration for the primary PMT "octagon". This configuration allows four-color analysis of FITC, PE, PerCP-Cy5.5 and PE-Cy7 off the primary 488 nm solid laser. The two "distal" detector slots in the signal path have no PMTs or dichroics.



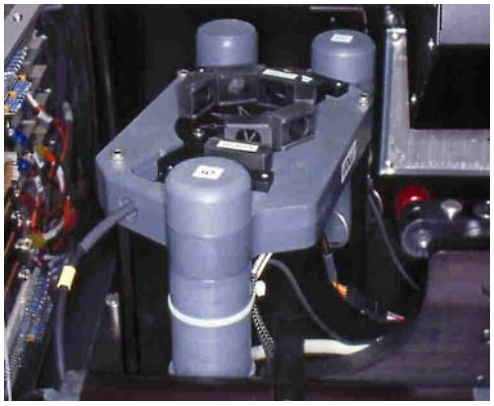
(Below). **Modified onfiguration for the detection of PE-Cy5.** Although the default configuration can be used for PE-Cy5 analysis (instead of PerCP-Cy5.5), it is not optimal. For a more optimal configuration, the PerCP-Cy5.5 bandpass filter can be changed to the PE-Cy5 670/14, and the dichroic in front of the detector changed to the 635 LP (custom fabricated by Omega Optical).

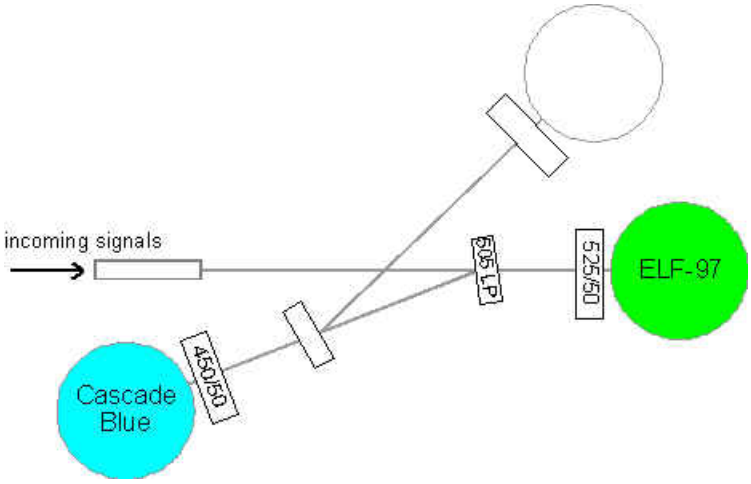


(Below). **Proposed configuration for six-color analysis with the primary 488 nm detector octagon.** Two additional PMTs can be added to the primary 488 nm octagon for six-color analysis. Filter and dichroics for PE-Texas Red and PE-Cy5.5 would be added to the configuration, and the side scatter, FITC and PE detector positions would be shifted to accommodate the additional detectors.

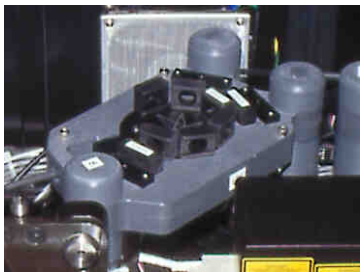


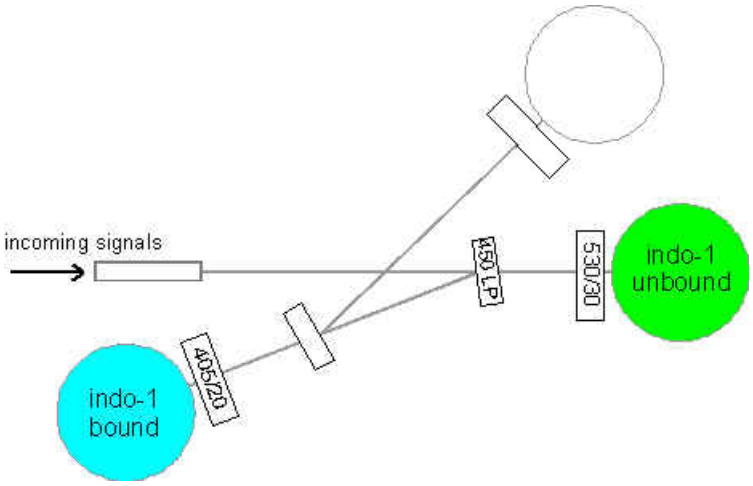
(Below). **Default configuration for the secondary violet diode detectors.** The violet laser diode beam occupies the secondary beam/signal position. *Left*, the secondary PMT "trigon". *Right*, the current default configuration for the secondary PMT trigon. This configuration allows simultaneous detection of two blue and green violet-excited fluorochromes, such as Cascade Blue and ELF-97 (illustrated). The "distal" detector slot in the signal path has no PMT or dichroic.





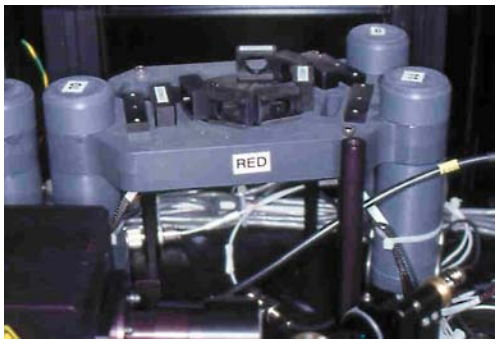
(Below). Default configuration for the third position UV detectors. The UV 355 nm solid state laser beam will occupy the third beam/signal position. *Left*, the third PMT "trigon". *Right*, the current default configuration for the third PMT trigon. This configuration allows simulaneous detection of two UV-excited fluorochromes, such as the ratiometric calcium probe indo-1 (illustrated). The "distal" detector slot in the signal path has no PMT or dichroic.

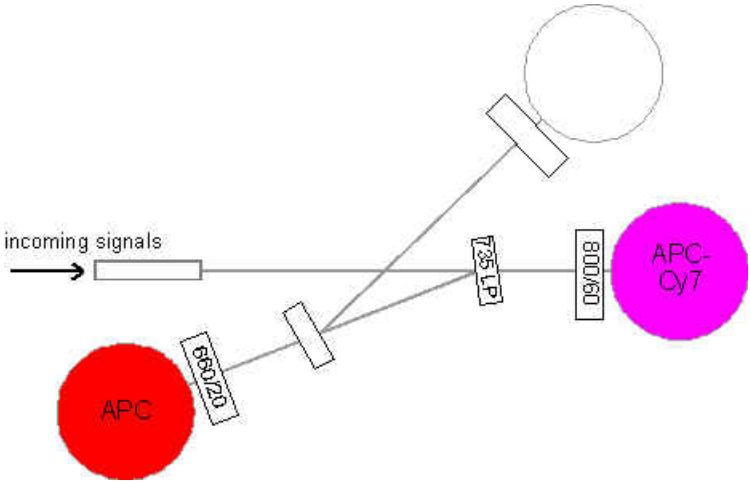




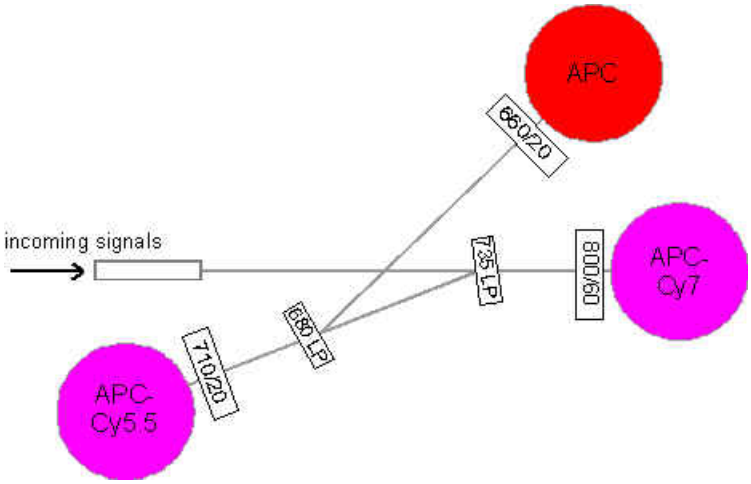
(Below). Default configuration for the fourth position red HeNe detectors. The 633 nm HeNe laser beam will occupies the fourth beam/signal position. *Left*, the fourth PMT "trigon". *Right*, the current default configuration for the fourth PMT trigon. This configuration allows simulaneous detection of two

red-excited fluorochromes, such as APC and APC-Cy7. The "distal" detector slot in the signal path has no PMT or dichroic in the default configuration.





(Below). **Addition of a third detector to the fourth position 633 nm detector trigon.** A third PMT can be added to this trigon, allowing simultaneous detection of APC, APC-Cy5.5 and APC.Cy7. Go here to see sample data.

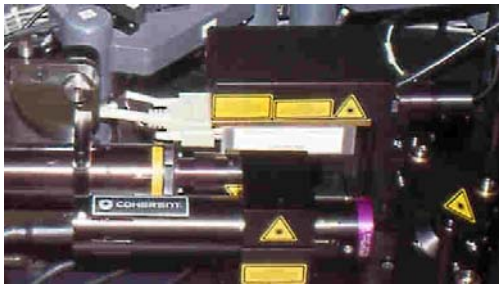


(Below). **LSR II dichroics.** The indicated custom fabricated dichroics were prepared for specific applications and have a 13 degree angle of incidence.

Dichroic	Use
450 LP	separation of indo-1 signals
505 LP	separation of FITC and side scatter or Cascade Blue and ELF-97
555 LP	separation of FITC and PE
595 LP	separation of PE and PE-Cy5 or PerCP-Cy5.5
610 SP	separation of Hoechst blue and red signals for SP analysis **CUSTOM**
635 LP	separation of PE-Texas Red and PE-Cy5 or Texas Red and APC **CUSTOM**
680 LP	separation of APC and APC-Cy5.5
695 LP	separation of PE-Cy5 and PE-Cy5.5 **CUSTOM**
735 LP	separation of APC-Cy7 from APC or APC-Cy5.5

(Below). **FACSVantage DiVa filters.** Although these filters are supplied with the instrument, any standard 25 mm bandpass filters can be substituted.

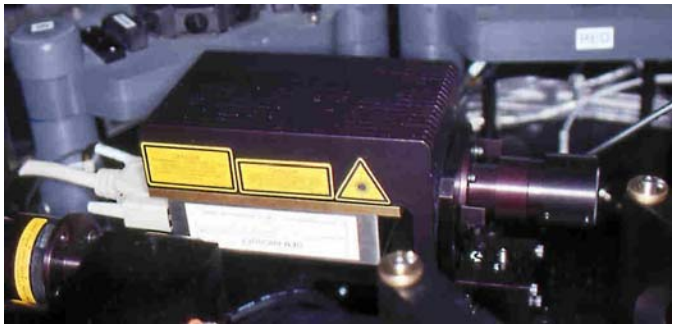
Filter	Use
405/20	indo-1 (bound Ca)
450/50	Cascade Blue, Pacific Blue, Alexa Fluor 350, DAPI, Hoechst dyes, SP analysis
488/10	side scatter, CFP
525/50	Cascade Yellow, ELF-97, GFP
530/30	FITC, Alexa Fluor 488
575/26	PE, YFP
610/20	PE-Texas Red, Texas Red, Alexa Fluor 594, DsRed
660/20	APC, Alexa Fluor 633
670/14	PE-Cy5, PE-Alexa Fluor 647, Alexa Fluor 647, SP analysis
695/40	PerCP-Cy5.5, PE-Cy5.5, PE-Alexa Fluor 680, APC-Alexa Fluor 680, APC-Alexa Fluor 700. Cy5.5, Alexa Fluor 680, Alexa Fluor 700
800/60	APC-Cy7, PE-Alexa Fluor 750, APC-Alexa Fluor 750


COHERENT

Solid state 488 nm excitation on the LSR II.

The LSR II uses a Coherent Sapphire 20 mW solid state 488 nm laser, replacing the air-cooled argon-ion gas lasers found on the original LSR and earlier flow cytometers. These lasers have minimal cooling requirements and a reputed to be highly stable; plasma tube glow and noise is also eliminated.

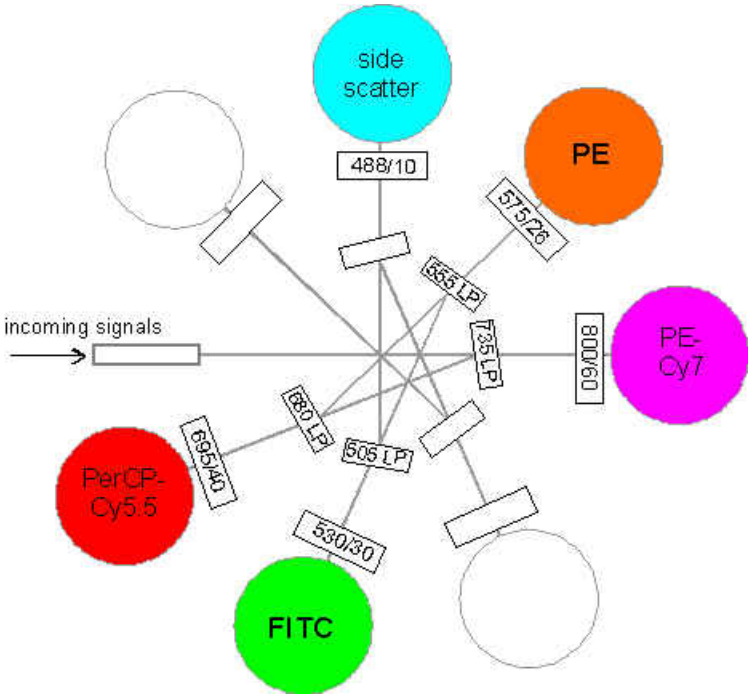
(Below). **The Coherent Sapphire 25 mW solid state 488 nm laser.** The laser is the silver unit in the bottom half of the photo; the black object on top of it is a (largely unnecessary) heat sink. The beam shaping optics (required to match the beam diameter of the violet diode laser) are aiming to the right.



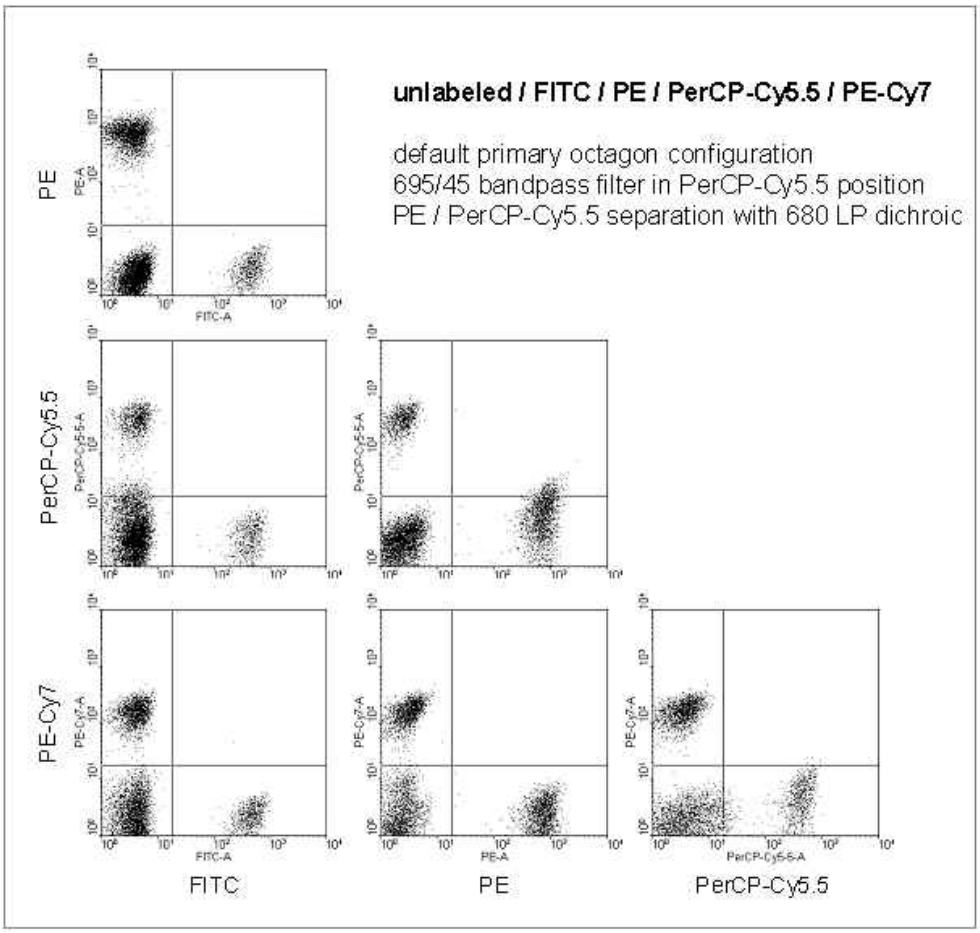
Single laser four-color analysis on the LSR II. The 488 nm laser "octagon" is currently equiped with five PMTs, four for fluorochrome detection and one for side scatter mesasurement. The unit can accomodate up to seven PMTs, six for fluorochrome analysis. The octagon uses a series of long pass dichroics to transmit and reflect incoming signals, "capturing" the longest fluorochrome signals (such as PE-Cy7) in the proximal PMTs and the shortest wavelength signals (such as FITC and side scatter) in the distal PMTs. The default configuration is set up for simultaneous FITC, PE, PerCP-Cy5.5 and PE-Cy7 analysis; the more common fluorochrome PE-Cy5 can also be substituted for PerCP-Cy5.5 with this configuration.

(Below). **"Octagon" configuration for the primary 488 nm detectors.** PMTs are contained in the vertical cylinders; filters and dichroics are visible in slots positioned around the center of the unit. The transmitting fiber optic enters the unit from the left.

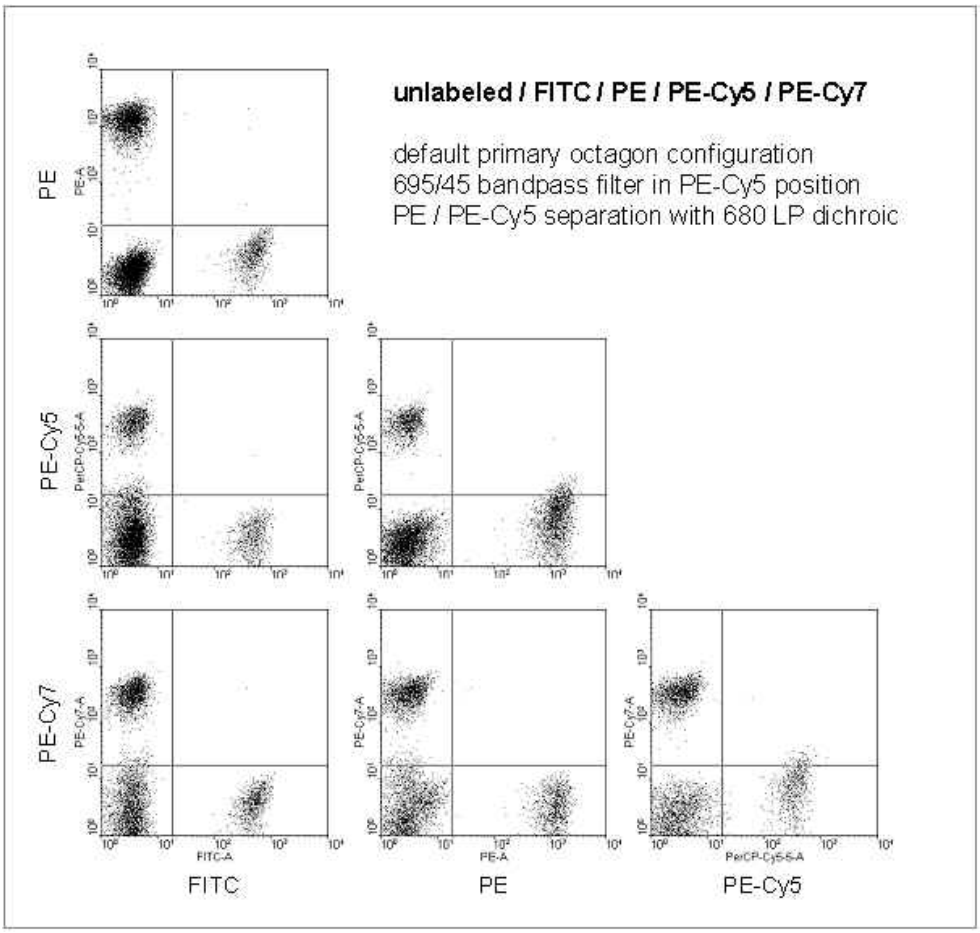
(Below). **The current default configuration for the primary PMT "octagon".** This configuration allows four-color analysis of FITC, PE, PerCP-Cy5.5 and PE-Cy7 off the primary 488 nm solid laser. The two "distal" detector slots in the signal path have no PMTs or dichroics.



(Below). **Four-color analysis of FITC, PE, PerC-Cy5.5 and PE-Cy7 using the default "octagon" configuration.** EL4 cells were labeled with biotin-conjugated CD44 followed by FITC, PE or PerCP-Cy5.5 and PE-Cy7-conjugated streptavidin, and a "cocktail" of unlabeled / FITC / PE / PerCP-Cy5.5 / PE-Cy7 labeled cells was analyzed using the above default configuration.

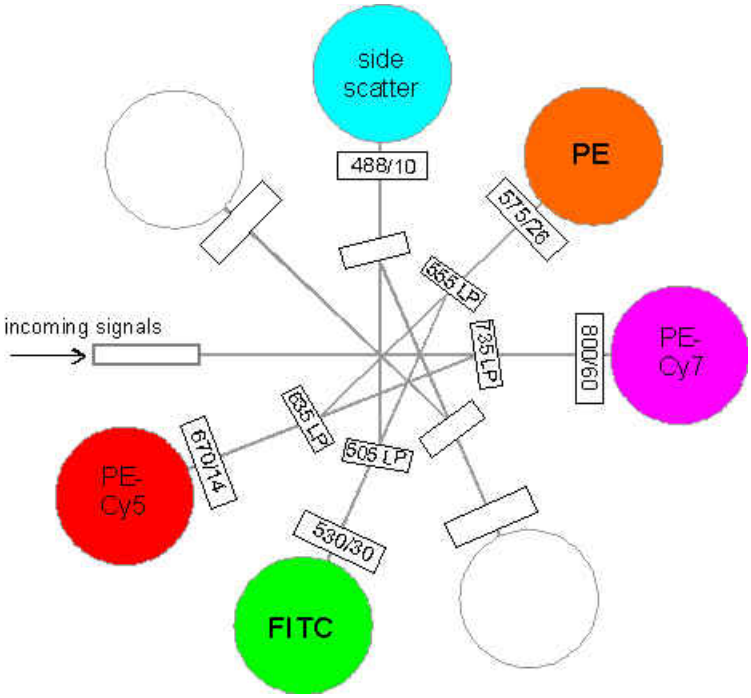


(Below). **Four-color analysis of FITC, PE, PE-Cy5 and PE-Cy7 using the default "octagon" configuration.** EL4 cells were labeled with biotin-conjugated CD44 followed by FITC, PE or PE-Cy5 and PE-Cy7-conjugated streptavidin, and a "cocktail" of unlabeled / FITC / PE / PerCP-Cy5.5 / PE-Cy7 labeled cells was analyzed using the above default configuration.

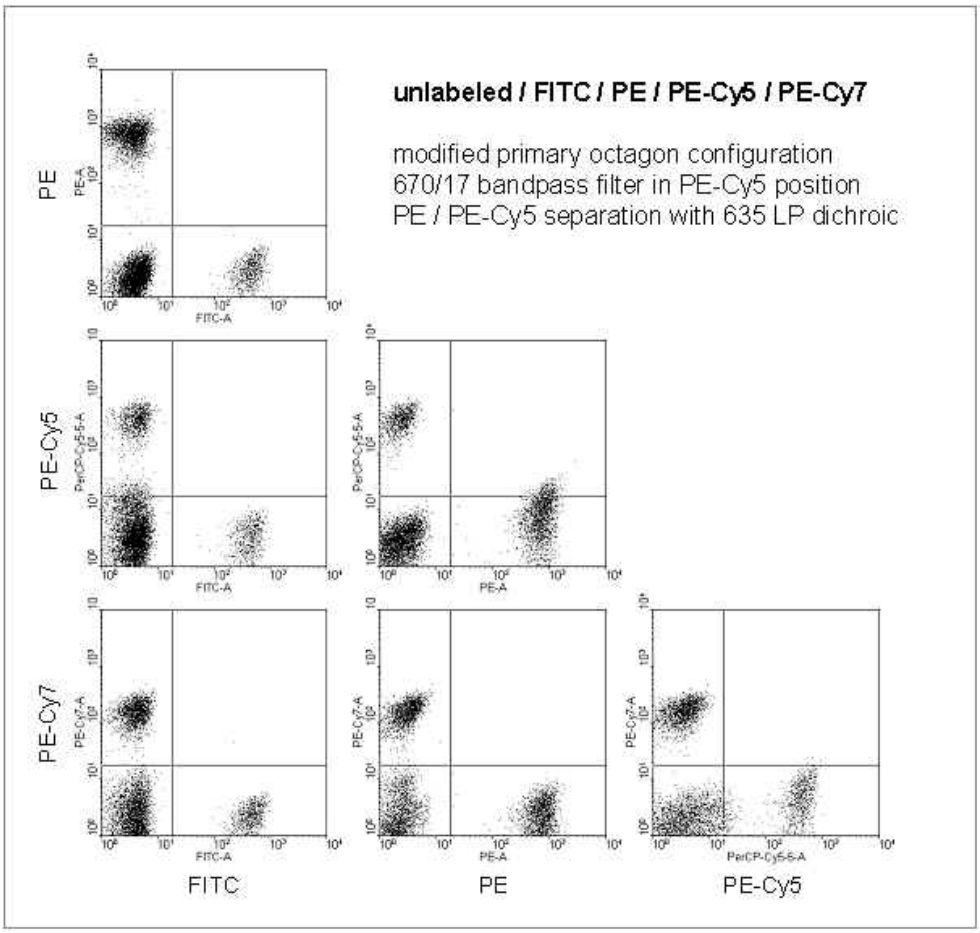


The octagon can be modified for better analysis of PE-Cy5 by inserting a 670/14 nm bandpass filter in front of the PE-Cy5 detector (in place of the 695/40 filter used for PerCP-Cy5.5) and replacing the corresponding 680 LP dichroic with a 635 LP (custom fabricated by Omega Optical).

(Below). **The modified configuration for the primary PMT "octagon".** The PerCP-Cy5.5 595/40 nm filter has been replaced with a 670/14 nm PE-Cy5 filter, and the corresponding dichroic has been replaced with a 635 LP. This configuration allows better four-color analysis of FITC, PE, PE-Cy5 and PE-Cy7 off the primary 488 nm solid laser.

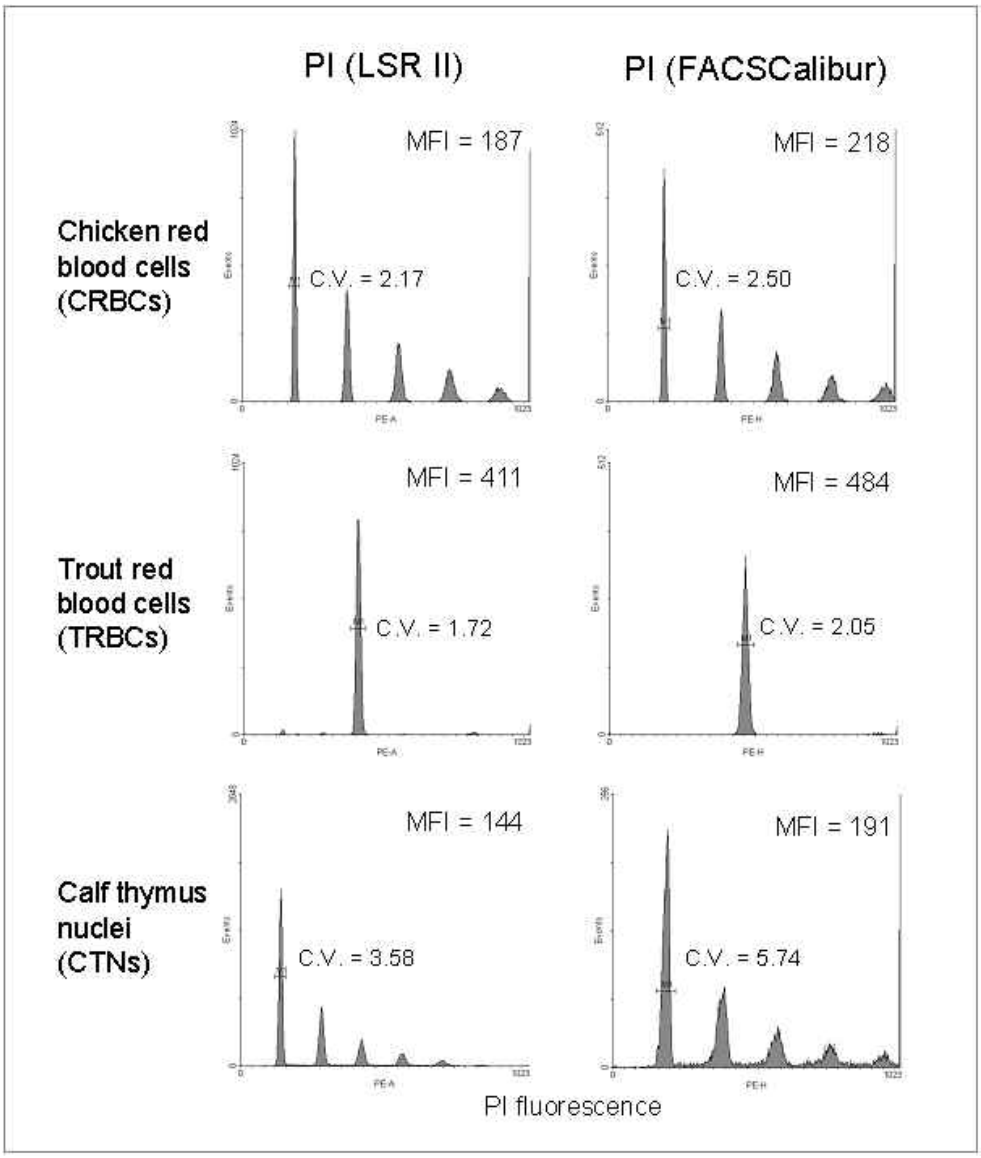


(Below). **Four-color analysis of FITC, PE, PE-Cy5 and PE-Cy7 using the modified "octagon" configuration.** EL4 cells were labeled with biotin-conjugated CD44 followed by FITC, PE or PE-Cy5 and PE-Cy7-conjugated streptavidin, and a "cocktail" of unlabeled / FITC / PE / PerCP-Cy5.5 / PE-Cy7 labeled cells was analyzed using the above modified configuration.

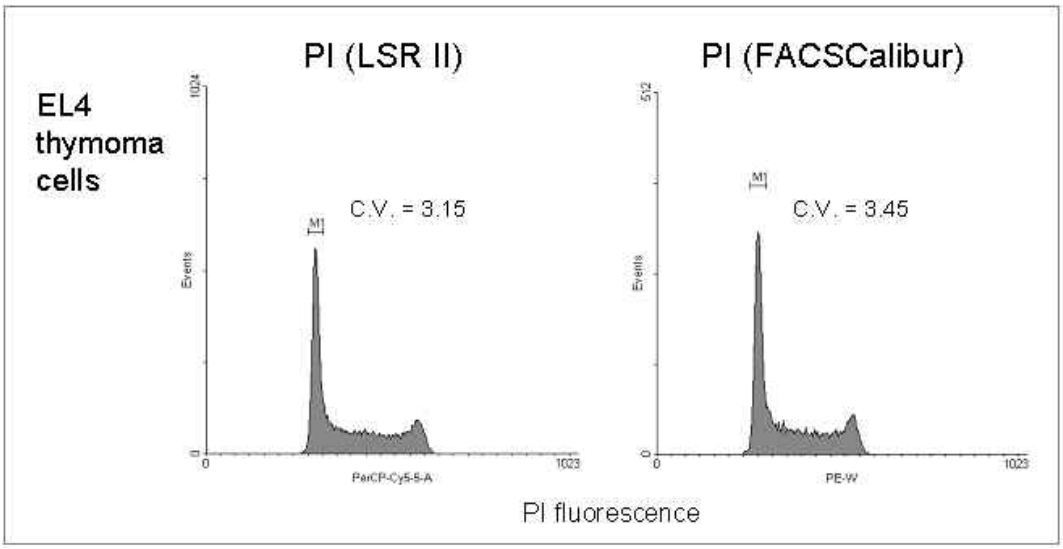


DNA content analysis on the LSR II. Measurement of cellular DNA content and cell cycle is traditionally carried out with the DNA binding dye propidium iodide (PI) on flow cytometers equipped with quartz flow cells. The good light collection efficiency and low numerical aperture of the LSR II collection optics suggests that it might be well-suited for DNA content analysis using PI. The LSR II was therefore tested for DNA content analysis using chicken RBCs, trout RBCs and calf thymus nuclei standards, as well as EL4 cells labeled with PI. The LSR II gave some improvement in DNA content peak C.V.s compared to the BD FACSCalibur, an indicator of cell cycle resolution.

(Below). **DNA content analysis with propidium iodide on the LSR II.** Chicken red blood cells (CRBCs), trout red blood cells (TRBCs) and calf thymus nuclei (CTNs) were labeled with PI at 50 ug/ml with simultaneous RNase treatmentand analyzed on the LSR II with the 488 nm laser and through the PE detector (left column). Cells were simultaneously analyzed on the BD FACSCalibur with 488 nm excitation and signal collection through the PE channel (right column). Mean fluorescent intensities and C.V.s for the singlet peaks are shown.



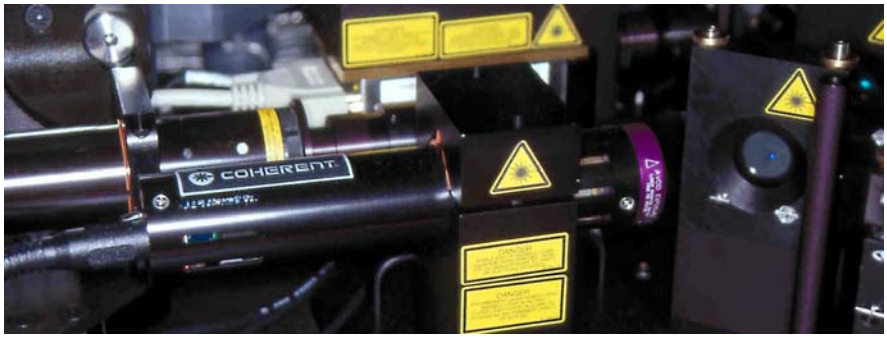
(Below). **DNA content analysis with propidium iodide on the LSR II.** EL4 cell nuclei were prepared with a solution of NP-40 in PBS and labeled with PI at 50 ug/ml with simultaneous RNase treatment, followed by analysis on the LSR II with the 488 nm laser and through the PE detector (left histogram). Cells were simultaneously analyzed on the BD FACSCalibur with 488 nm excitation and signal collection through the PE channel (right histogram). Mean fluorescent intensities and C.V.s for the singlet peaks are shown.


EL4
thymoma
cells
PI (LSR II)
C.V. = 3.15
M1
Events
1024
0
0
1023
PerCP-Cy5-5-A
PI (FACSCalibur)
C.V. = 3.45
M1
Events
512
0
0
1023
PE-W
PI fluorescence

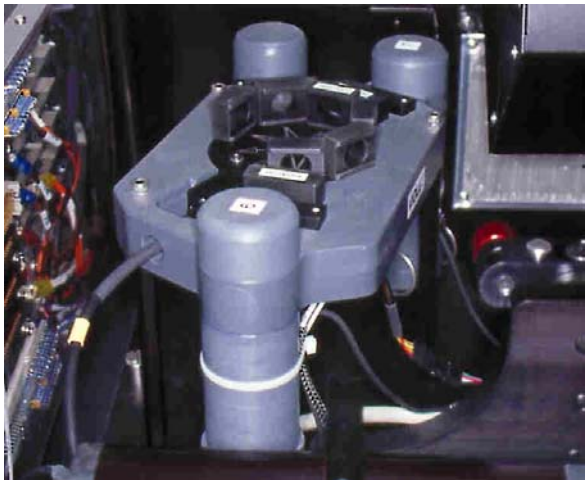
Violet laser diode excitation on the BD Biosciences LSR II.

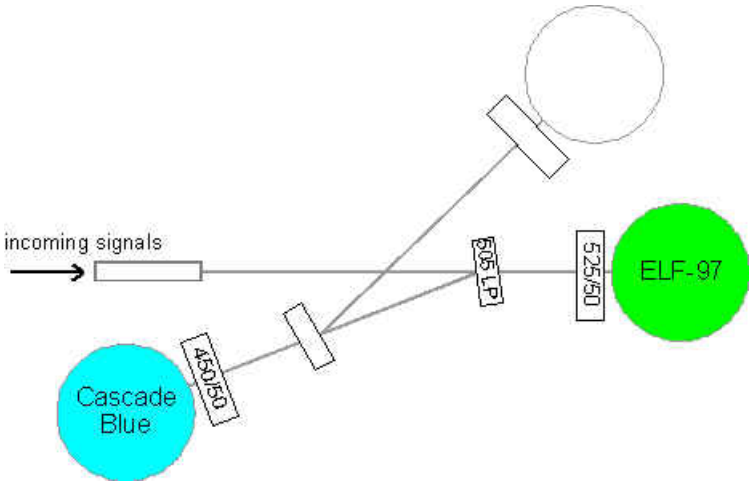
The LSR II is equipped with a Coherent VioFlame 408 nm violet diode laser (VLD) in the second position emitting at 25 mW. The VLD is useful for the excitation of the immunophenotyping fluorochromes Cascade Blue and Pacific Blue, Cyan Fluorescence Protein (CFP), and the fluorogenic alkaline phosphatase substrate ELF-97. Incorporation of the VLD into the LSR II allows the addition of violet excited flurochromes for advanced multicolor immunophenotyping, and analysis of multiple fluorescence proteins and fluorescence resonance energy transfer (FRET) using CFP. The VLD and the LSR II collection optics also give surprisingly high-resolution cell cycle analysis using the UV-excited DNA binding dye DAPI. A PMT trigon allows the analysis of up to two violet-excited fluorochromes simultaneously in the default configuration.

(Below). The **Coherent VioFlame violet diode laser on the LSC II.** The VLD has independent steering optics for easier laser alignment.



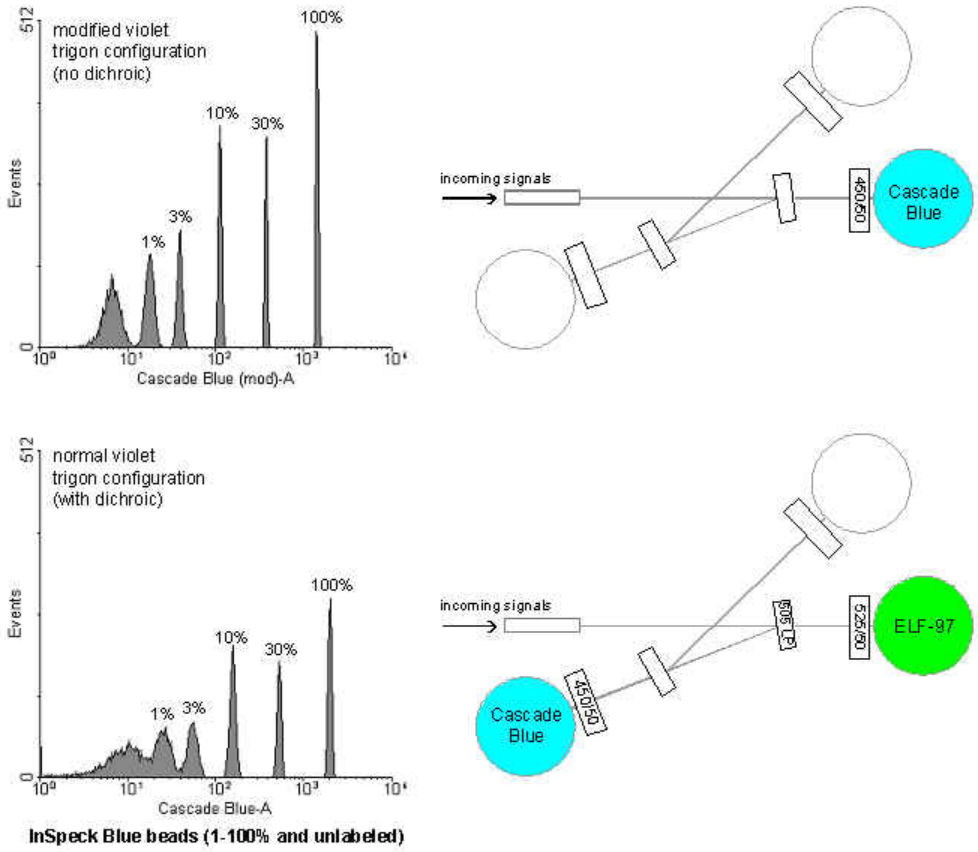
(Below). Default configuration for the secondary violet diode detectors. The violet laser diode beam occupies the secondary beam/signal position. *Left*, the secondary PMT "trigon". *Right*, the current default configuration for the secondary PMT trigon. This configuration allows simultaneous detection of two blue and green violet-excited fluorochromes, such as Cascade Blue and ELF-97 (illustrated). The "distal" detector slot in the signal path has no PMT or dichroic in the default configuration.





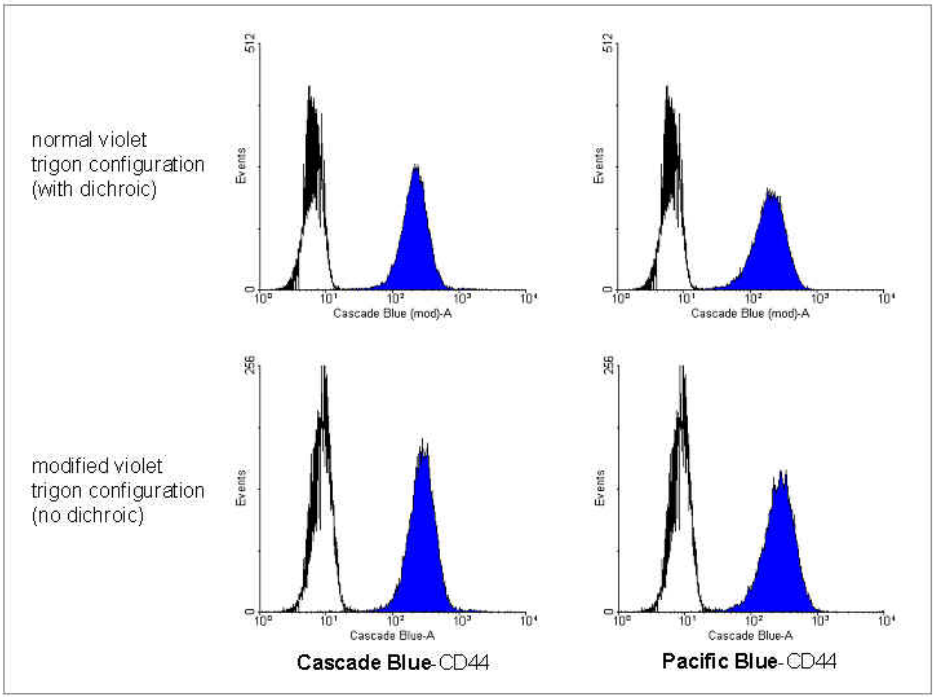
Although the LSR II can detect up to two violet-excited fluorochromes simultaneously in the default position, single fluorochrome detection is best carried out by removing the 505 LP dichroic and sending the signal into the proximal PMT position, since reflection off the dichroic reduces the signal intensity somewhat.

(Below). Analysis of linearity beads in default and modified violet diode trigon configuration. Molecular Probes InSpeck Blue beads were analyzed on the LSR II using VLD excitation and detection through the Cascade Blue 450/50 nm filter. The bead "cocktails" (containing a arbitrary 100% intensity bead population, and populations with decreasing intensity down to 1%, plus unlabeled) were analyzed either with the 505 LP dichroic removed and the 450/50 nm filter placed in front of the the proximal PMT (top row) or with default trigon configuration (bottom row).

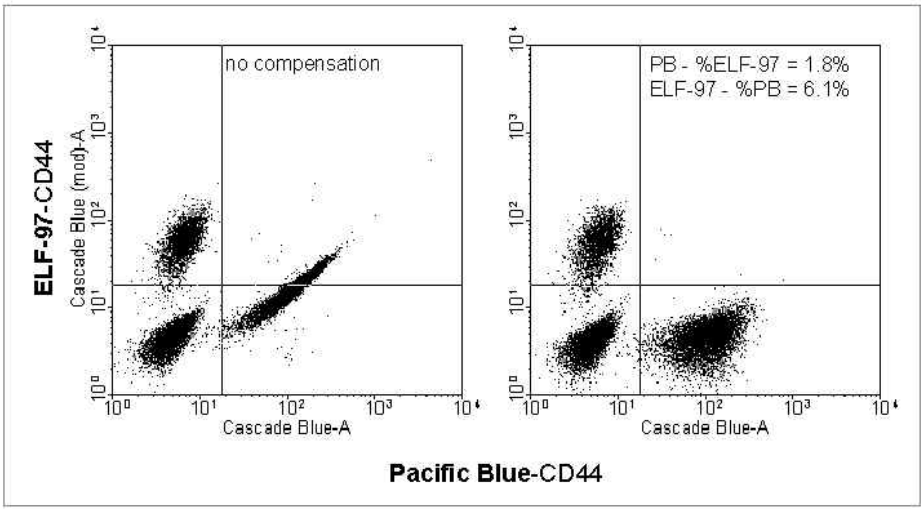


Detection of Cascade Blue and Pacific Blue on the LSR II. Cascade Blue and Pacific Blue are violet-excited fluorochromes from Molecular Probes that can be conjugated to antibodies and other proteins. We have previously shown that they are well-excited with low-power violet laser diode sources (see the data here). These fluorochromes also work well on the LSR II. In addition, Cascade Blue or Pacific Blue can be combined with ELF-97 immunolabeling. ELF-97 is a fluorogenic alkaline phosphatase substrate that labels AP-conjugated reagents with a bright green fluorescent precipitate. The emission spectra of ELF-97 is similar to Cascade Yellow, making ELF-97 a potentially useful fluorochrome for polychromatic flow cytometry with UV or violet laser sources.

(Below). **Cascade Blue and Pacific Blue detection on the LSR II.** EL4 cells were labeled with biotin-conjugated CD44 followed by Cascade Blue or Pacific Blue -conjugated strepavidin. "Cocktails" of unlabeled and labeled cells were then analyzed on the LSR II with the VLD. As demonstrated above, slightly better sensitivity in single color analysis was achieved by removing the intervening dichroic.



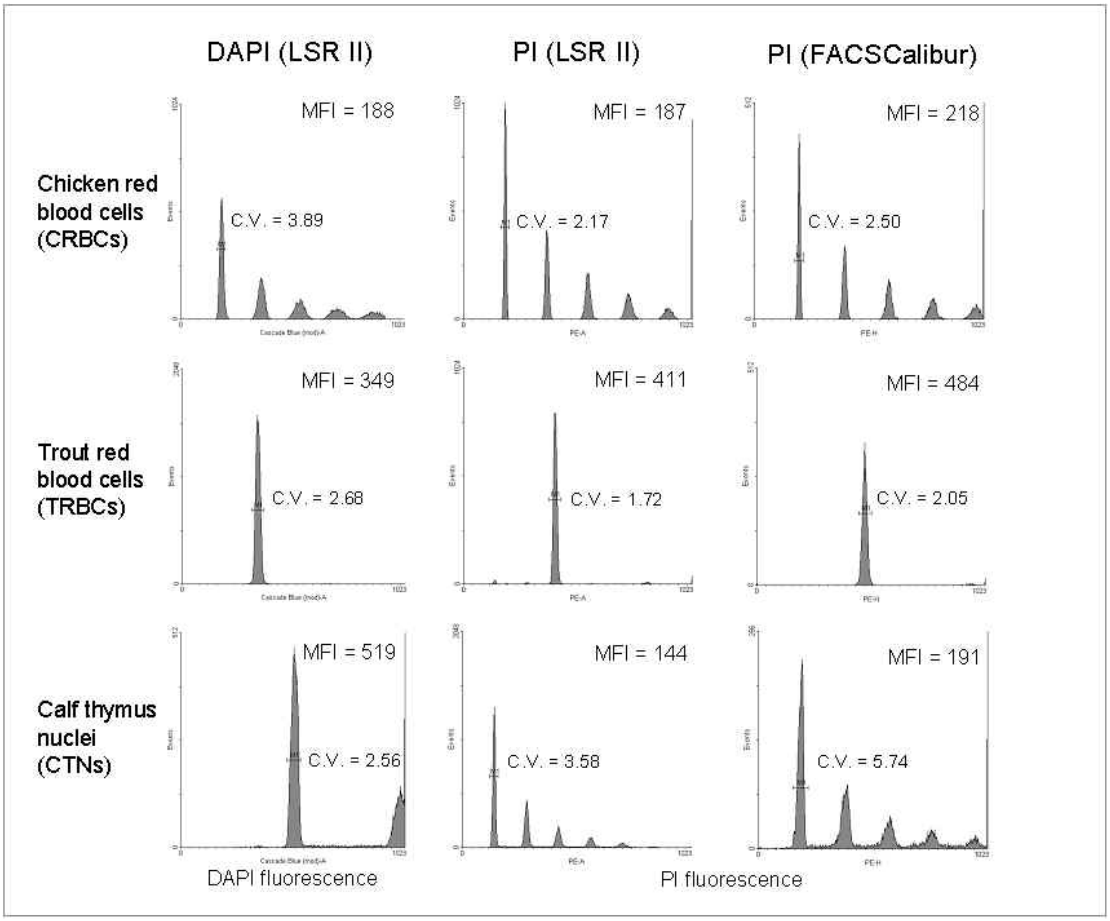
(Below). **Combined Pacific Blue and ELF-97 detection on the LSR II.** EL4 cells were labeled with biotin-conjugated CD44 followed by alkaline phosphatase (AP) or Pacific Blue -conjugated strepavidin. AP-labeled cells were then labeled with ELF-97. "Cocktails" of unlabeled and labeled cells were then analyzed on the LSR II with the VLD (uncompensated cytogram on the left, compensated on the right). Both Cascade Blue and Pacific Blue have good spectral compatibility with ELF-97, requiring minimal compensation.



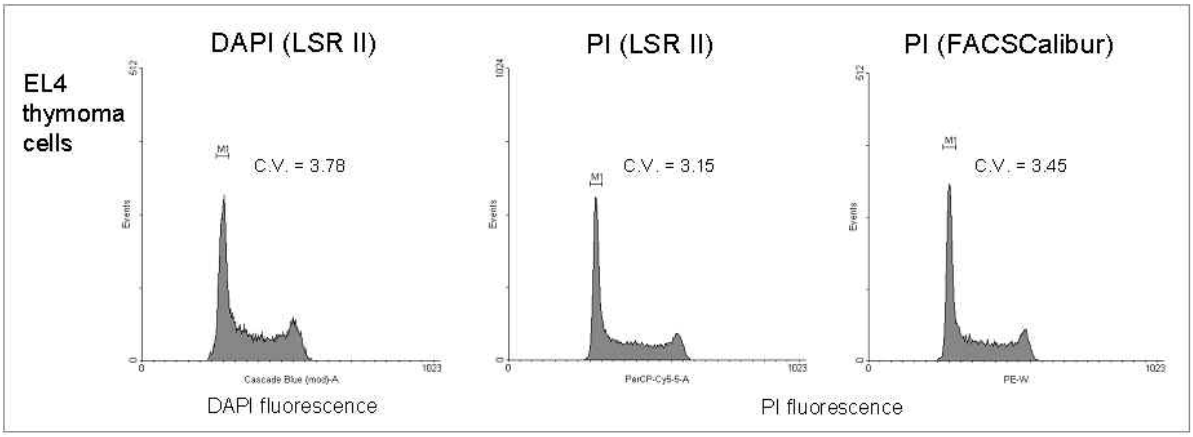
DNA content analysis with DAPI and the VLD on the LSR II. DAPI is one of the most useful DNA binding dyes for cell cycle analysis, exhibiting relatively low non-specific binding, particularly to RNA. Unfortunely, DAPI excitation requires an ultraviolet laser source, an uncommon item on flow cytometers with quartz flow cells.

Nevertheless, several laboratories have observed that DAPI can be reasonably well-excited with violet laser diodes, making the current LSR II configuration potentially useful for measuring DAPI DNA content. The LSR II was therefore tested for DNA content analysis using chicken RBCs, trout RBCs and calf thymus nuclei standards, as well as EL4 cells. DNA content resolution and peak C.V.s were still somewhat poorer than those observed with propidium iodide analysis both on the LSR II itself and on the FACSCalibur, but were within acceptable limits. The future addition of the solid state UV laser to the LSR II bench should ultimately give excellent DNA content resolution for DAPI, based on these preliminary results.

(Below). **DNA content analysis with DAPI on the LSR II.** Chicken red blood cells (CRBCs), trout red blood cells (TRBCs) and calf thymus nuclei (CTNs) were labeled with DAPI at 5 ug/ml and analyzed on the LSR II with the VLD and the violet diode trigon set up with the dichroic removed and the 450/50 nm in front of the proximal dichroic. Cells were simultaneously analyzed through the LSR II PE channel with 488 nm excitation, and on the BD FACSCalibur with 488 nm excitation and signal collection through the PE channel. Mean fluorescent intensities and C.V.s for the singlet peaks are shown.



(Below). **DNA content analysis with DAPI on the LSR II.** EL4 cell nuclei were prepared with a solution of NP-40 in PBS and labeled with DAPI at 5 ug/ml, followed by analysis on the LSR II with the VLD and the violet diode trigon set up with the dichroic removed and the 450/50 nm in front of the proximal dichroic. Cells were simultaneously analyzed through the LSR II PE channel with 488 nm excitation, and on the BD FACSCalibur with 488 nm excitation and signal collection through the PE channel. Mean fluorescent intensities and C.V.s for the singlet peaks are shown.

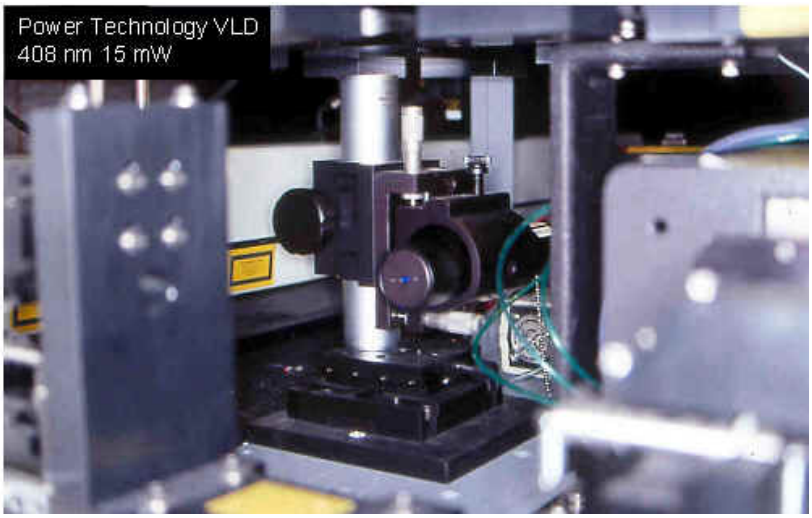


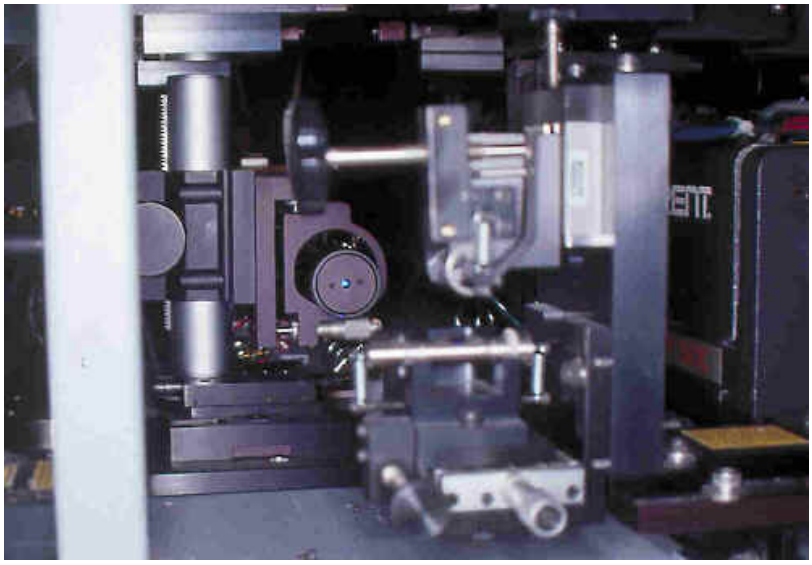
DNA content resolution was lower than with propidium iodide, but surprisingly good given the lower power of the VLD.

Comparison of the Coherent VioFlame VLD on the LSR II to the Power Technology VLD on the FACSVantage DiVa. We have previously mounted a Power Technology 30 mW 408 nm violet diode laser on our FACSVantage DiVa; this configuration gives similar sensitivity to our krypton-ion 407 nm water-cooled laser on the sorter (click here for information and data). Below we've compared the Coherent VioFlame mounted on the LSR II to our Power Technology VLD/FACSVantage DiVa instrument configuration. Sensitivity for Cascade Blue, Pacific Blue and ELF-97 were found to be similar between the two lasers and instruments. Sensitivity for CFP fluorescence was better of the LSRII - this is mainly due to the reduced laser light spillover from the low-power Sapphire laser on the LSR II compared to the more powerful water-cooled argon on the FACSVantage, resulting in lower backgrounds and better signal-to-noise ratios on the LSR II. Detection of both Pacific Blue and CFP on both instruments require a 488 nm notch filter sandwiched with the 463/50 or 485/22 nm emission filters to reduce laser noise and maximize instrument sensitivity.

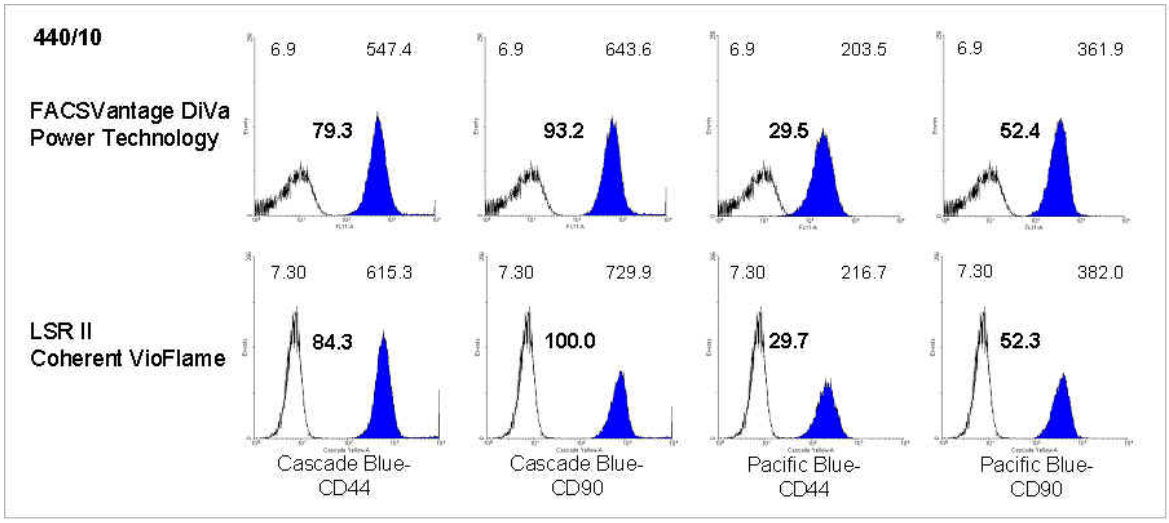
The same bandpass filters were used for all comparisons. Violet-excited fluoroscence on the LSR II was collected through the most proximal PMT with no intervening dichroic (see above for diagram). Fluorescence on the FACSVantage was also collected with no intervening dichroic.

(Below). **Power Technology VLD laser mounted on the FACSVantage DiVa.** The VLD is mounted in place of the krypton-ion laser that normally occupies this position on the instrument. Details on mounting the instrument can be found here.

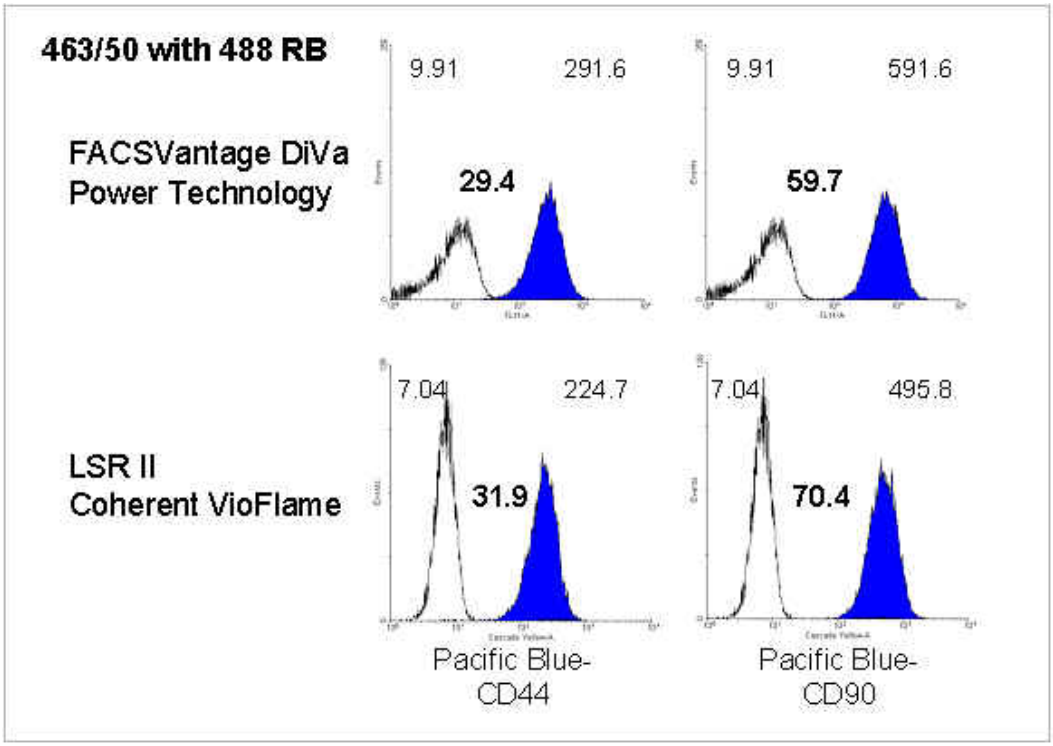




(Below). **Cascade Blue and Pacific Blue detection on the FACSVantage DiVa or LSR II with VLD excitation.** EL4 cells were labeled with biotin-conjugated CD44 followed by Cascade Blue or Pacific Blue -conjugated strepavidin. Cells were then analyzed on either the FACSVantage DiVa with the Power Technology VLD (top row), or on the LSR II with the Coherent VioFlame (bottom row). Detection was through the same 440/10 nm filter. Median fluorescence intensity (MFI) values are shown at the top of the histograms, and the ratio is in boldface.

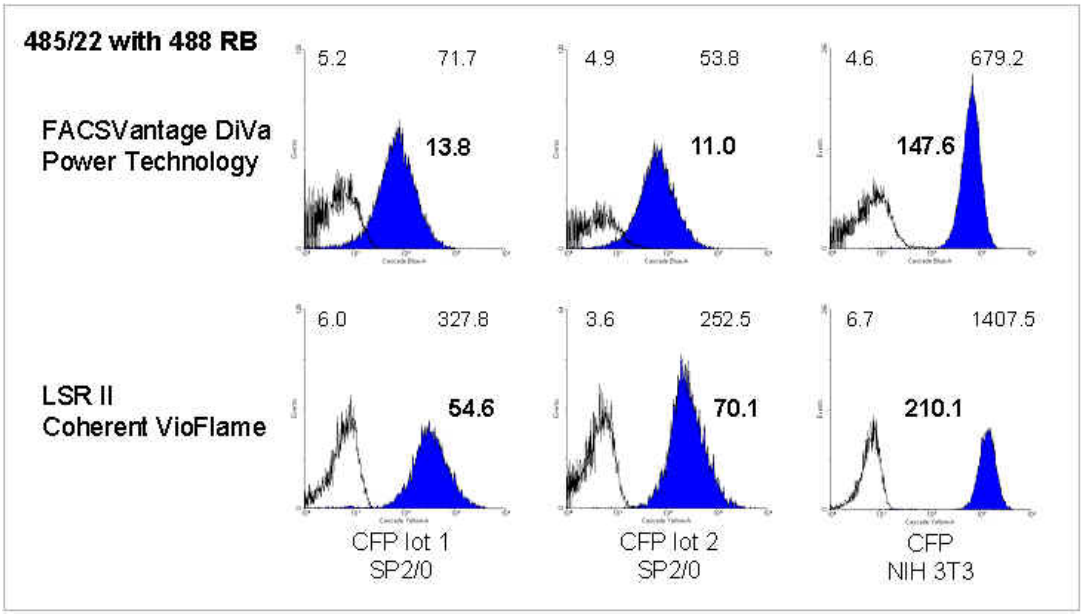


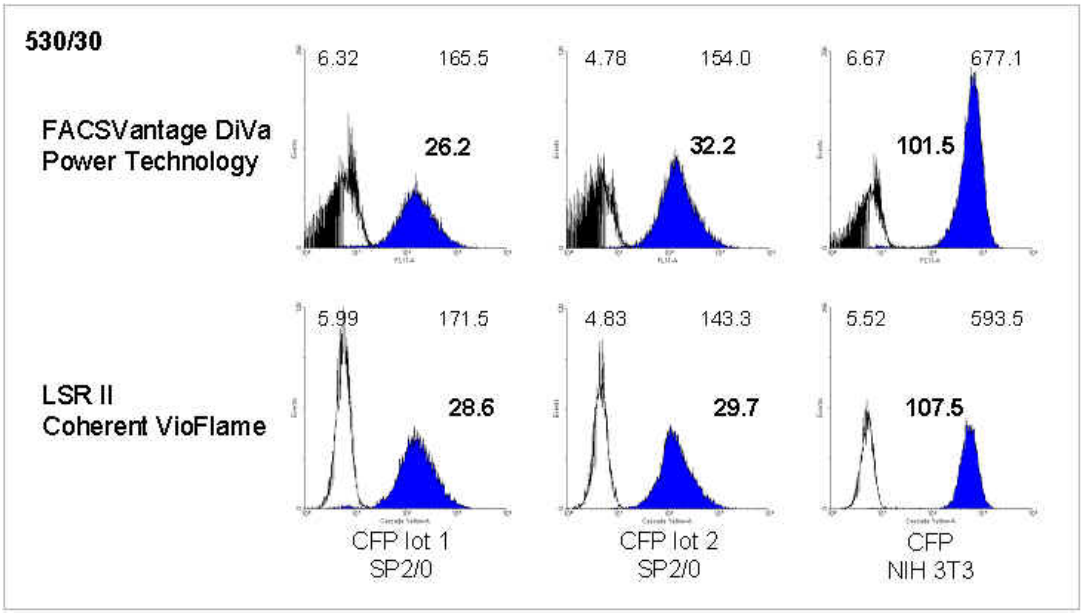
(Below). **Pacific Blue detection. on the FACSVantage DiVa or LSR II with VLD excitation, with a 463/50 nm filter.** EL4 cells were labeled with biotin-conjugated CD44 followed by Pacific Blue - conjugated strepavidin. Cells were then analyzed on either the FACSVantage DiVa with the Power Technology VLD (top row), or on the LSR II with the Coherent VioFlame (bottom row). Detection was through a 463/50 nm filter sandwiched with a 488 nm notch filter to block 488 nm laser emission.



(Below). **Cyan Fluorescence Protein detection on the FACSVantage DiVa or LSR II with VLD excitation, with a 463/50 nm filter.** SP2/0 cells (left and middle histograms) or NIH 3T3 cells (right histograms) expressing enhanced CFP were analyzed on either the FACSVantage DiVa with the Power Technology VLD, or on the LSR II with the Coherent VioFlame, through either a 485/22 nm filter with 488 nm notch filter (top panels), or a 530/30 nm filter (bottom panels). Detection sensitivity was lower on the FACSVantage with the 485/22 nm / 488 nm RB filter combination due to the higher 488 nm laser power

and resultant spillover, as indicated by the 530/30 nm analysis, which showed similar sensitivity between instruments.



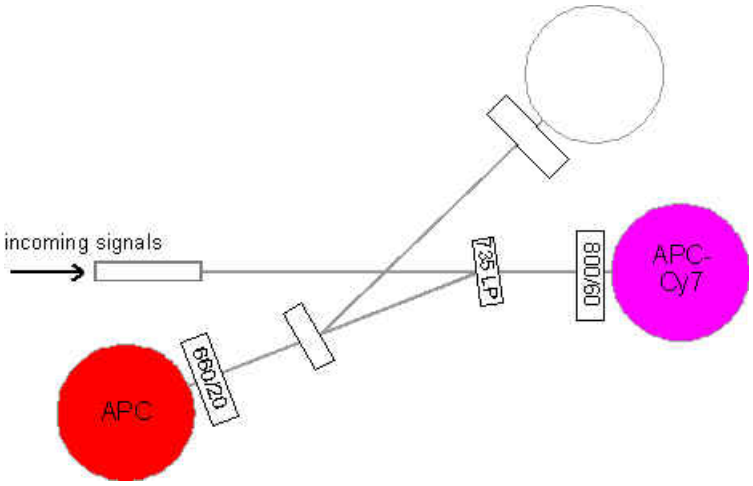


So, the LSR II was similar in sensitivity to the FACSVantage DiVa for violet laser diode excitation for Cascade Blue and ELF-97. Fluorochromes requiring an emission filter overlapping the 488 nm laser line (including Pacific Blue and CFP) were analyzed with greater sensitivity on instruments with lower 488 nm laser power like the LSR II, although inclusion of a 488 nm notch filter largely obviated this difference.

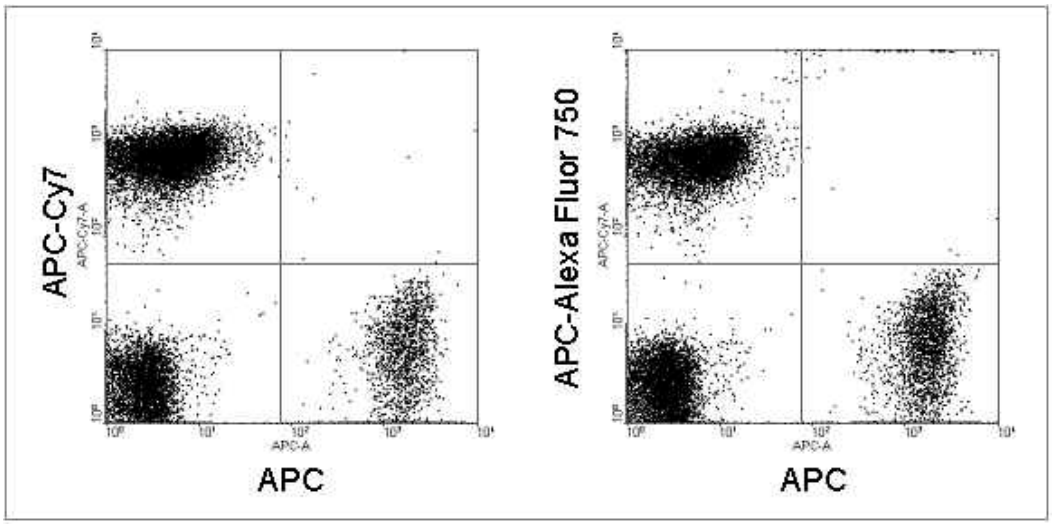
HeNe 633 nm excitation on the LSR II.

The LSR II is equipped with a JDS Uniphase red 633 nm helium-neon laser emitting at 17 mW in the fourth laser position. This laser is more than adequate for exciting a variety of red-excited flurochromes, including APC and its tandem conjugates (including APC-Cy5.5, APC-Alexa Fluor 680, APC-Alexa Fluor 700, APC-Cy7 and APC-Alexa Fluor 750), Cy5, Alexa Fluor 633, Alexa Fluor 647, and the DNA binding dye TO-PRO-3. The red HeNe has a corresponding "trigon" with a default configuration of two PMTs; a third has been added to our instrument. In the default configuration, the LSR II can analyze APC and APC-Cy7, APC and APC-Cy5.5, or APC-Cy5.5 and APC-Cy7 simultaneously. With three PMTs, simultaneous analyis of APC / APC-Cy5.5 / APC-Cy7 is possible.

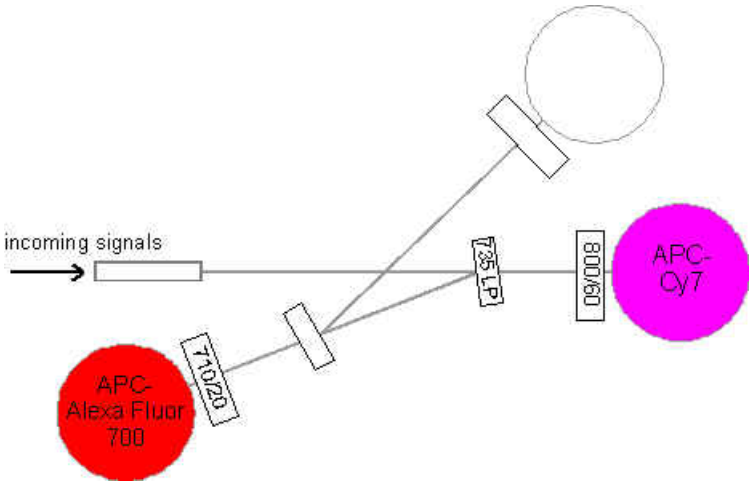
(Below). **HeNe laser trigon configuration.** The LSR II HeNe laser trigon in the default configuration, with filters for APC and APC-Cy7 split with a 735 LP dichroic.



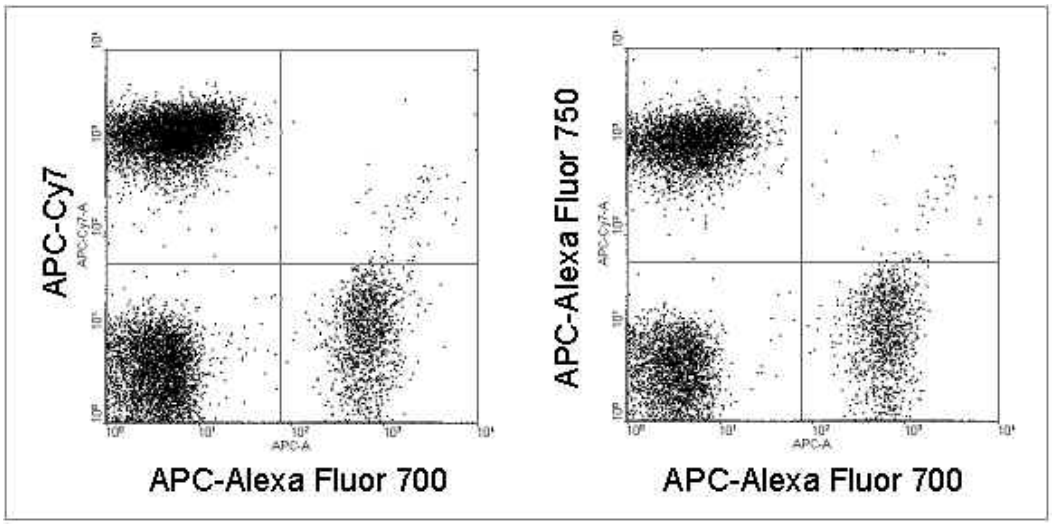
(Below). **Two-color analysis of APC and APC-Cy7 or APC-Alexa Fluor 750 with the HeNe 633 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC, APC-Cy7 or APC-Alexa Fluor 750-conjugated streptavidin, and "cocktails" of unlabeled, APC, APC-Cy7 or APC-Alexa Fluor 750 labeled cells were analyzed using the red HeNe laser.



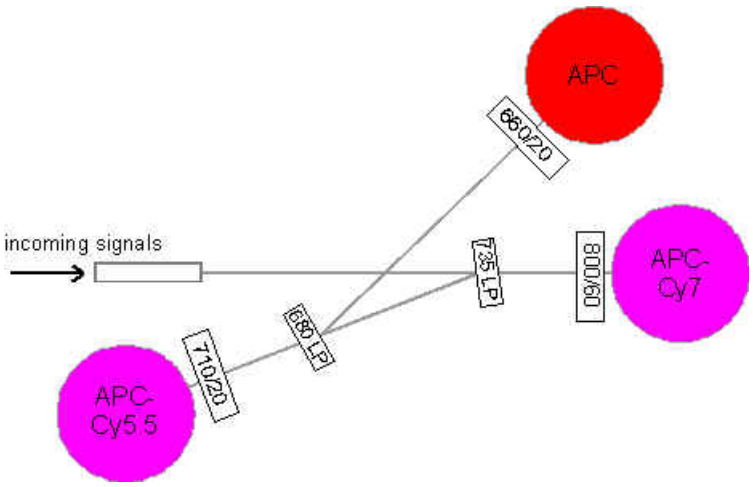
(Below). **HeNe laser trigon configuration modified for APC-Cy5.5 (or APC-Alexa Fluor 680 or APC-Alexa Fluor 700) and APC-Cy7 (or APC-Alexa Fluor 750) .** The LSR II HeNe laser trigon in modified configuration, with a 710/20 nm filter for APC-Cy5.5 and a signal split with the 735 LP dichroic.



(Below). **Two-color analysis of APC-AF700 and APC-Cy7 or APC-Alexa Fluor 750.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC-Alexa Fluor 700, APC-Cy7 or APC-Alexa Fluor 750-conjugated streptavidin, and "cocktails" of unlabeled, APC, APC-Cy7 or APC-Alexa Fluor 750 labeled cells were analyzed using the red HeNe laser.



(Below). **HeNe laser trigon configuration modified for APC, APC-Cy5.5 (or APC-Alexa Fluor 680 or APC-Alexa Fluor 700) and APC-Cy7 (or APC-Alexa Fluor 750) .** The LSR II HeNe laser trigon in modified configuration, with a third PMT, a 710/20 nm filter for APC-Cy5.5 and the APC / APC-Cy5.5 split made with a 680 or 695 LP dichroic.



This configuration is being evaluated now.

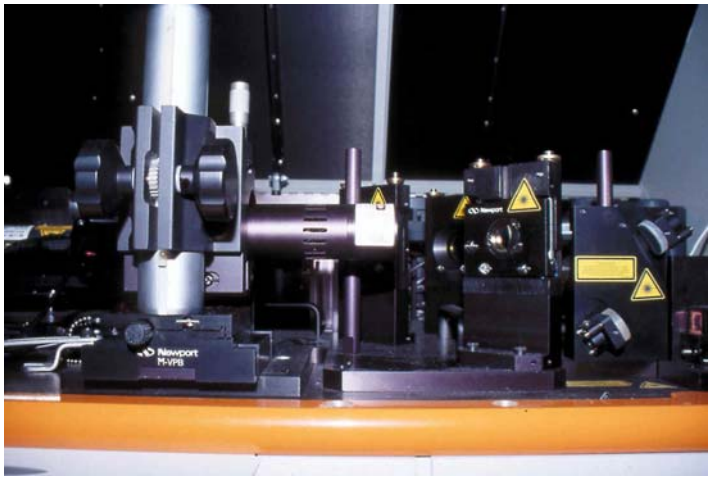
The red HeNe can be replaced with a yellow 594 nm HeNe laser for up to four-color analysis with Texas Red. Go here to see our initial evaluation.

Near UV laser diodes (NUVLDs) on the BD LSR II.

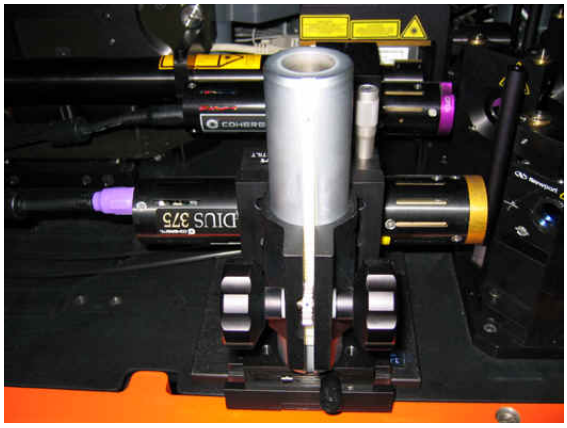
Near-UV GaN diode lasers (NUVLDs) are recent developments in diode laser technology. These lasers emit in the 370 to 380 nm range, approaching true ultraviolet emission. Like more widely available blue and violet diodes, they are small, air-cooled and are becoming commercially available in increasing power levels. These lasers have the potential to provide a source of near-ultraviolet excitation in a package far smaller and lower in maintenance than other UV lasers, such gas sources and triple-pumped Nd-YAG lasers. While violet diodes (395 to 410 nm) can substitute for certain UV applications (such as DAPI cell cycle), laser sources closer to true UV are likely to provide improved cell cycle analysis resolution. In addition, Hoechst 33342 side population (SP) analysis is poorly resolved by violet excitation; near-UV sources may provide better SP resolution, closer to that obtained with traditional UV-emitting gas lasers. NUVLDs are also likely to make good excitation sources for UV-excited immunophenotyping fluorochromes like Alexa Fluor 350 and Marina Blue, as well as recently developed Quantum Dots. To test the utility of NUVLDs for flow cytometry, several NUVLDs were mounted on our BD LSR II and evaluated for their ability to excite a variety of UV fluorochromes.

Incorporation of NUVLDs onto the BD LSR II. Four NUVLDs were mounted on the LSR II: a Power Technology 372 nm emitting at 1.5 mW, a Power Technology 370 nm emitting at 8 mW, a Coherent Radius 372 nm emitting at 8 mW, and a Point Source Ltd. 374 nm emitting at 10 mW. These lasers were mounted in the front-most laser position on our LSR II (in the UV position) and their beams aligned to the violet laser pinhole (the second signal path) on the LSR II. Power levels were confirmed with a Melles Griot broadband power meter. Laser and mounts are shown below. Lasers were aligned using Polyscience 2 micron yellow-green microspheres.

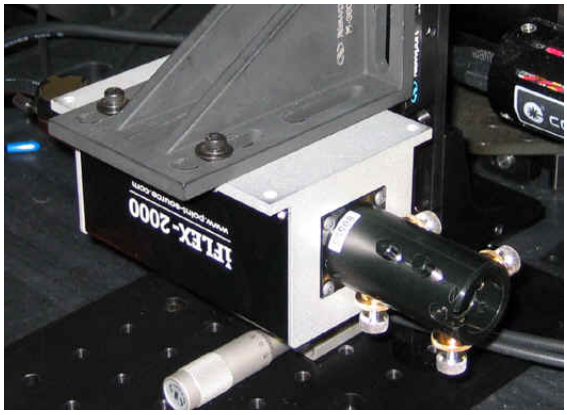
(Below). **Power Technology 370 nm 8 mW laser.**



(Below). **Coherent 372 nm 8 mW laser.**

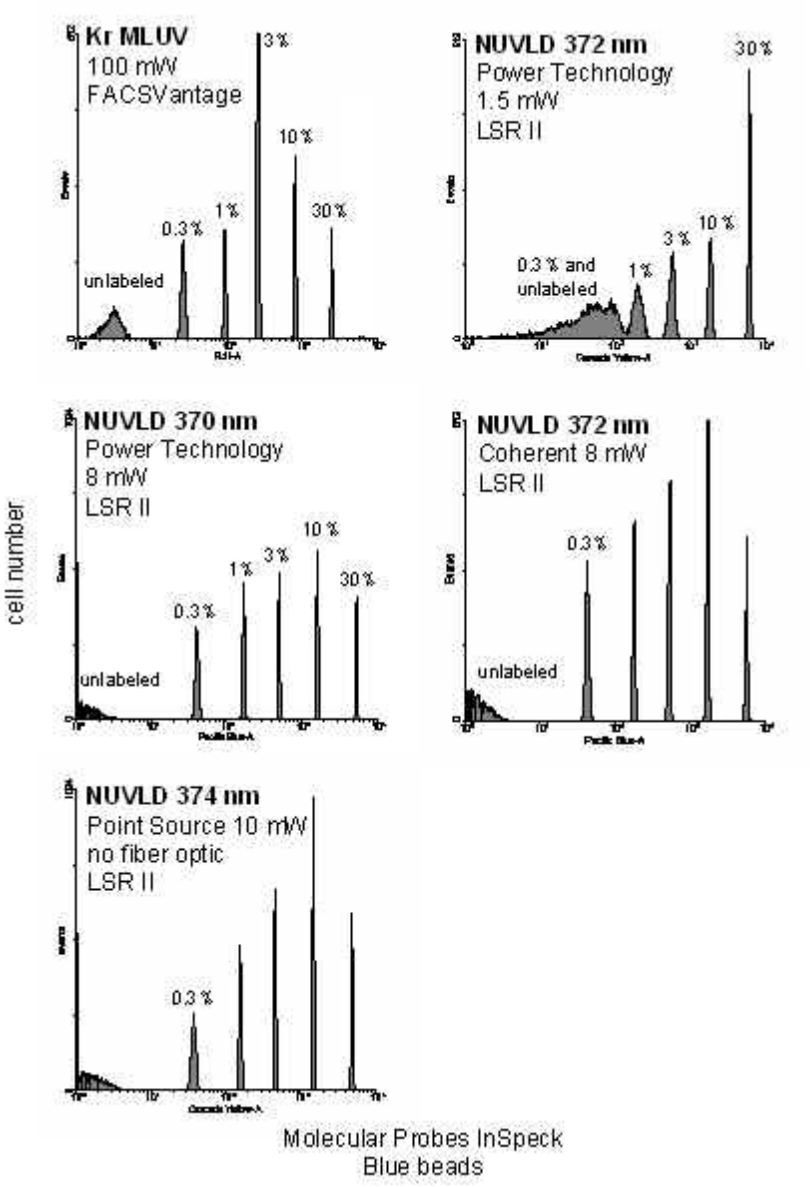


(Below). **Point Source Ltd. 374 nm 10 mW laser.** Shown and used here without a fiber optic.



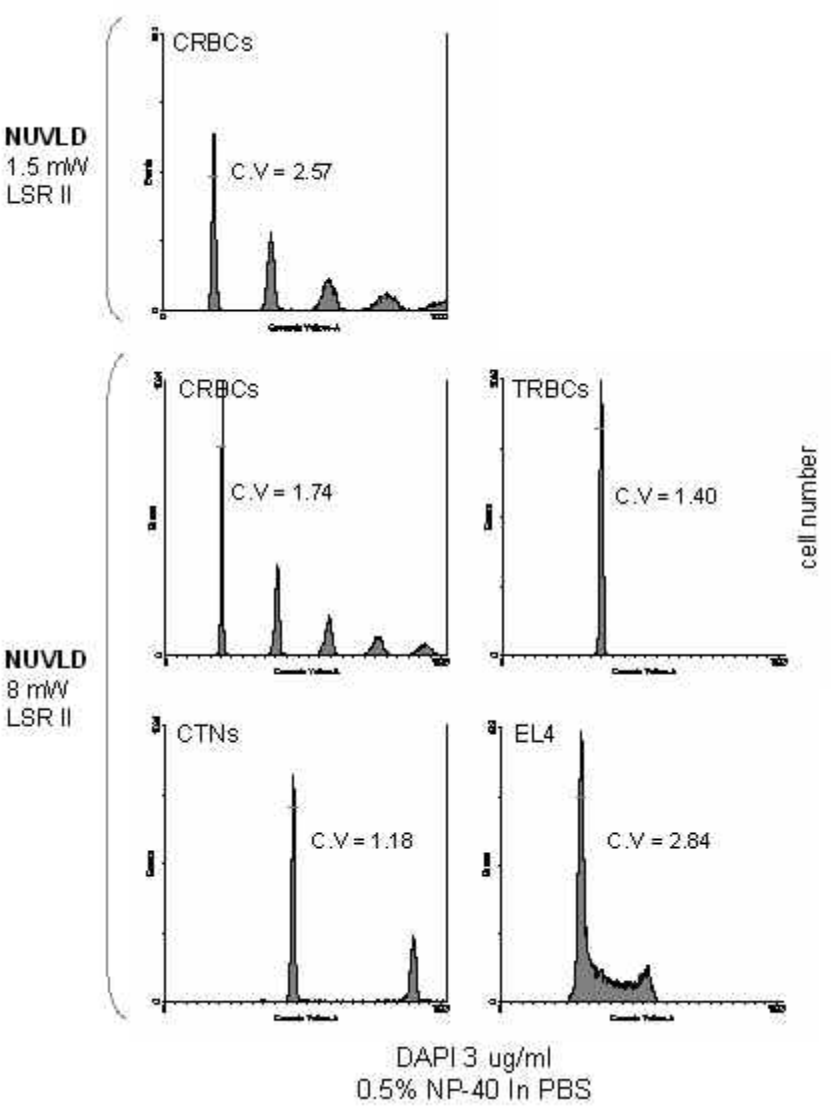
Alignment and sensitivity assessment. Laser alignment and resulting signal sensitivity was measured using Molecular Probes InSpeck Blue microspheres. For comparison purposes, the same microspheres were analyzed on a FACSVantage DiVa equipped with a krypton-ion laser emitting in the multiline UV mode at 100 mW. As shown below, the NUVLD emitting at 1.5 mW gave reduced sensitivity for the dimmest bead populations compared to the gas laser; however, the more powerful versions (8-10 mW) gave sensitivity levels comparable to or better than the gas source, easly distinguishing the dimmest bead population from the unlabeleds. As prevously observed with violet diodes, the combination of low-power diode source and a cuvette-confined sample stream gave results comparable to a gas laser emitting at a much higher power level on a stream-in-air instrument. ***All NUVLD 8-10 mW sources performed comparably.***

(Below). **Sensitivity assessment of NUVLD sources on the BD LSR II.** Molecular Probes InSpeck Blue microspheres were analyzed on the BD LSR II equipped with the indicated laser source.



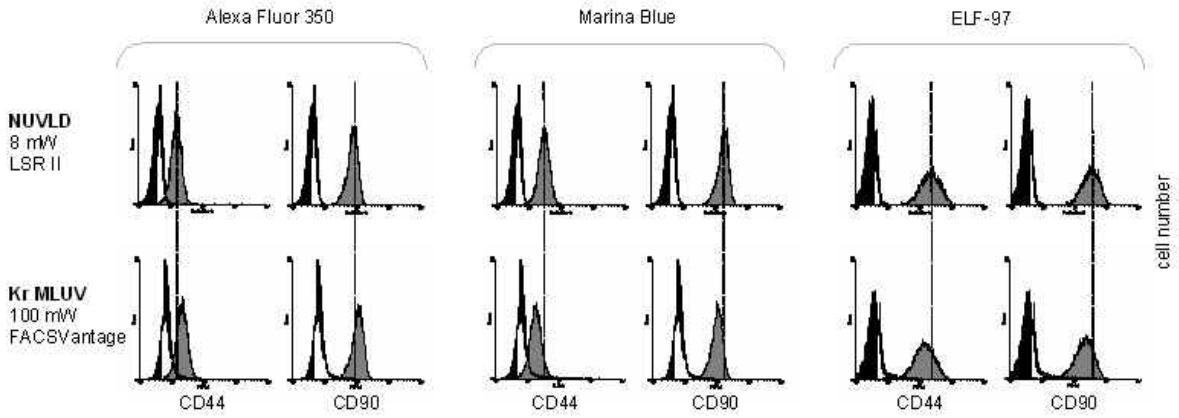
DAPI DNA content analysis. The ability of the NUVLDs to carry out DAPI DNA cell cycle was then assessed. Chicken red blood cell (CRBC), trout red blood cell (TRBC) and calf thymus nuclei (CTN) DNA standards labeled with DAPI were analyzed, as well as EL4 mouse thymoma cell nuclei. The 1.5 mW source gave acceptable DNA resolution, comparable to that obtainable with propidium iodide; however, the 8-10 mW versions were a significant improvement, giving peak C.V.s well below 2.0 for all DNA standards tested. Resolution was better than that previously observed for a more powerful violet diode source on the same instrument (see data here).

(Below). **DNA content analysis using NUVLDs on the BD LSR II.** Top histogram, CRBCs analyzed with the NUVLD 1.5 mW. Bottom four histograms, CRBCS, TRBCs, CTNs and EL4 mouse thymoma nuclei analyzed with an NUVLD 8 mW. Peak C.V.s are indicated by each peak.

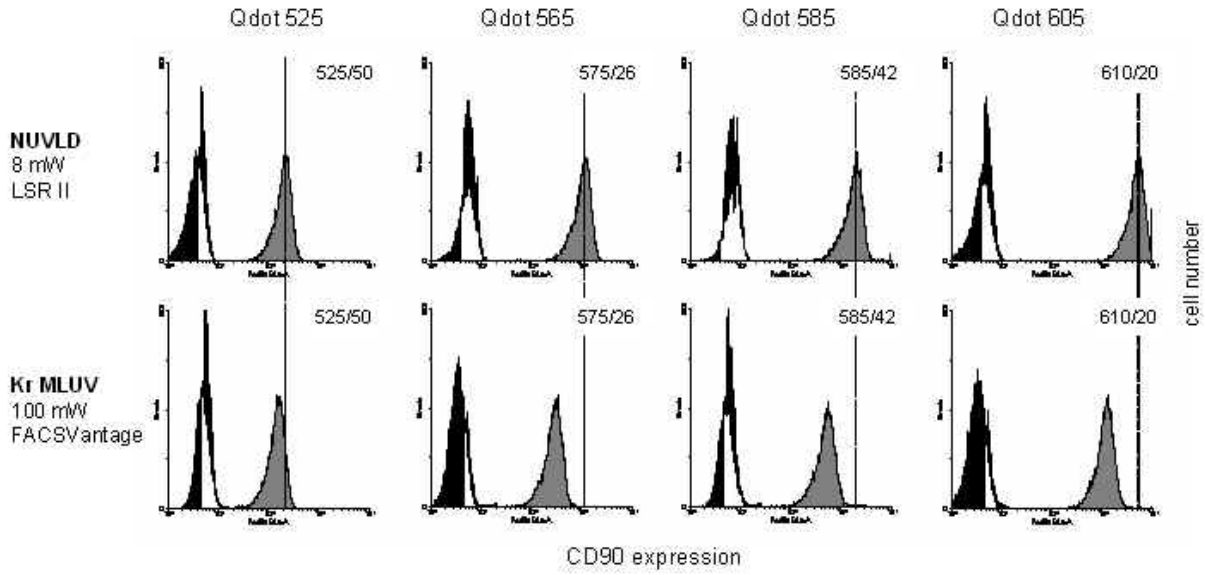


Immunophenotyping fluorochromes. The UV-excited immunophenotyping fluorochromes Alexa Fluor 350 and Marina Blue (both structurally based on the coumarin probe 7-aminomethylcoumarin or AMCA) are useful if somewhat dim probes for multicolor analysis. The fluorogenic alkaline phosphatase substrate ELF-97 is a much brighter UV-excited probe that can be used in same cases as an immunophenotyping label. NUVLDs in the 8-10 mW range gave sensitivity levels for these probes comparable to the gas laser on the FACSVantage DiVa.

(Below). **Immunophenotyping probe excitation with NUVLDs.** EL4 cells were labeled with either biotin-conjugated anti-CD44 or CD90 followed by streptavidin-conjugated Alexa Fluor 350 or Marina Blue, or streptavidin-conjugated alakline phosphatase followed by the ELF-97 substrate. Cells were then analyzed on either the LSR IIwithone of the NUVLD sources (top row), or the FACSVantage DiVa with the krypton-ion MLUV laser (bottom row).



Quantum dots. Quantum dots, encapsulated complexes of inorganic salts, are proving to be very useful fluorescent probes for a variety of applications. Since these probes are particularly well-excited by shorter wavelength lasers, we tested them with the NUVLD sources. In contrast to the low molecular weight probes shown above, sensitivity for Qdot fluorescence was better on the LSR II with all the 8-10 mW NUVLD sources, compared to the FACSVantage with the gas laser.



References

Telford, W.G. Small lasers in flow cytometry (invited book chapter). In Flow Cytometry, 2nd Edition, Humana Press, London, UK.

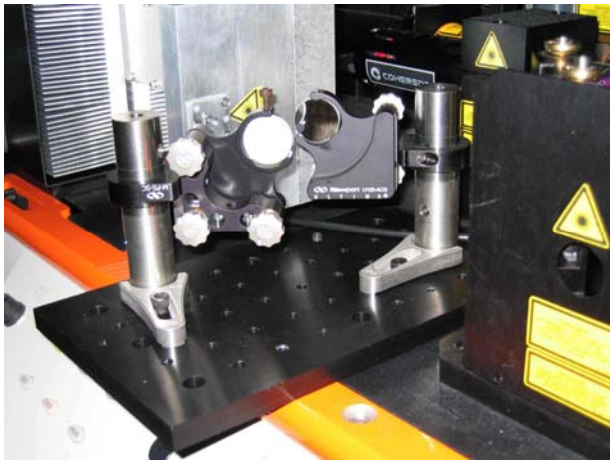
Telford, W.G. Analysis of UV-excited fluorochromes by flow cytometry using near-UV laser diodes. In press, Cytometry.

Frequency-tripled Nd-YAG UV laser on the BD LSR II.

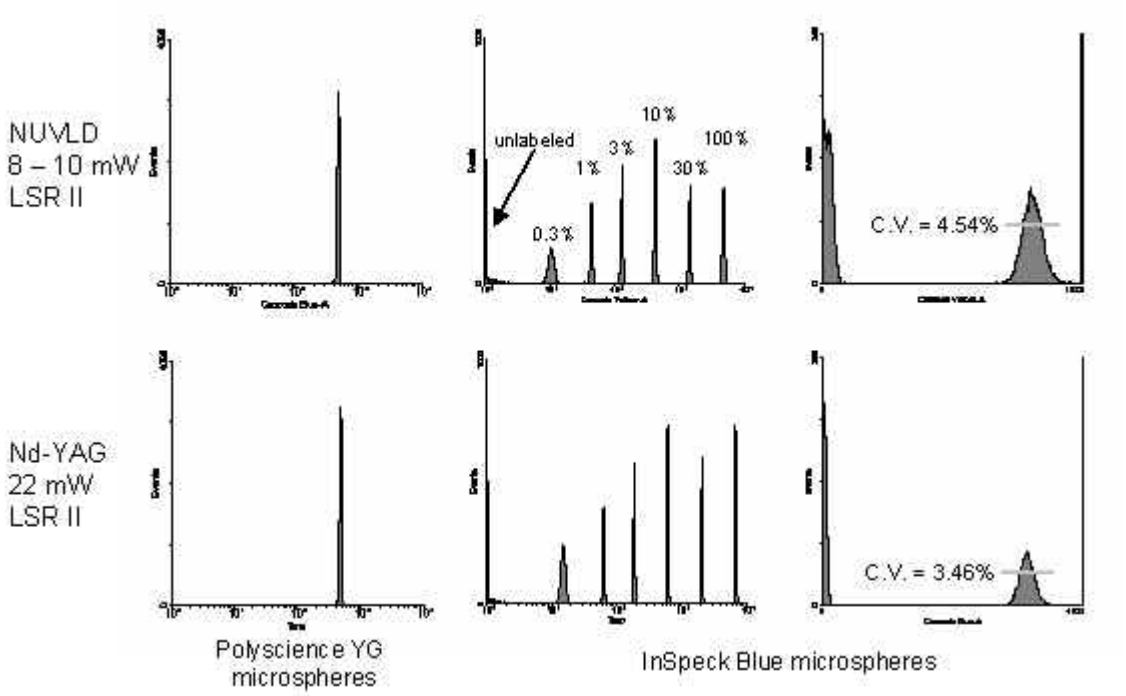
Lightwave Electronics, in collaboration with BD Biosciences, recently developed a frequency-tripled Nd-YAG solid state ultraviolet laser for the BD LSR II. Although technically a pulsed laser source, this laser has a sufficiently short pulse interval to operate essentially as a quasi-CW laser, allowing its use on a flow cytometer. Unlike most commercially developed Nd-YAG UV sources, this laser is of relatively low power (~20 mW), applicable for use on a cuvette-equipped flow cytometer. As a UV source, the Nd-YAG gave excellent results using our microsphere sensitivity and resolution standards, slightly better than that seen with our NUVLD source. It gave Hoechst 33342 side population comparable to the NUVLD source, and good indo-1 performance (an application not possible with the NUVLD source).

(Below). **Lightwave Electronics Nd-YAG UV laser, temporary installation.** The mode-locked UV laser was mounted temporarily on the BD LSR II prior to permanent installation, allowing laser alignment with two steering UV-compatible steering mirrors (detail in the lower photo). In this install, the laser was aligned to the third PMT trigon (normally used with the violet laser diode).

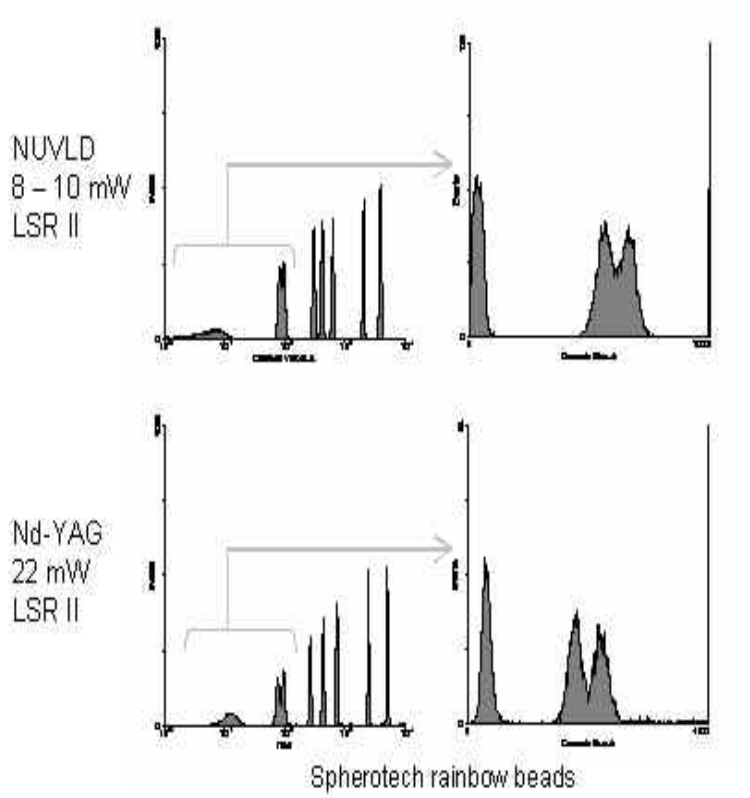
(Below). **Details on the steering mirrors.**



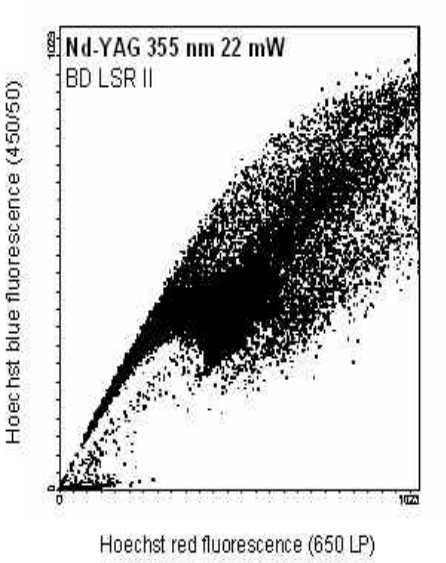
(Below). **Analysis of microsphere sensitivity/resolution standards with the Nd-YAG laser.** ***Left histograms***, Polyscience 2 micron yellow-green microspheres were analyzed on the BD LSR II with either a NUVLD (374 nm, 8 mW) or the Nd-YAG (355 nm, 22 mW). At the same PMT gain settings and filter configuration, the fluorescence signal was roughly equivalent for both laser sources. ***Middle histograms***, Molecular Probes InSpeck Blue microspheres were analyzed using either the NUVLD or the Nd-YAG. Both lasers gave excellent resolution of all seven differentially labeled bead populations. ***Right histograms,*** When the dimmest (0.3% and unlabeled InSpeck beads were analyzed with both lasers with linear scaling, the Nd-YAG laser gave slightly better resolution based on the peak C.V. of the dimmest bead population.



(Below). **Analysis of microsphere sensitivity/resolution standards with the Nd-YAG laser.** ***Left histograms,*** Spherotech Rainbow 8-population microspheres were analyzed on the BD LSR II with either a NUVLD (374 nm, 8 mW) or the Nd-YAG (355 nm, 22 mW) through a 450/50 nm filter. All eight populations were resolvable with both lasers, although the Nd-YAG gave slightly better resolution of the separation between the two dimmest populations ***(right histograms)***.



(Below). **Hoechst 33342 side population analysis with the Nd-YAG laser.** Mouse bone marrow was labeled with Hoechst 33342 at 5 ug/ml for 90 minutes and analyzed on the BD LSR II using the Nd-YAG laser. The Hoechst side population was easily resolvable with this laser source, comparable to the NUVLD.



(Below). **Indo-1 analysis on the BD LSR II with the Nd-YAG laser.** Mouse EL4 cells were loaded with the ratiometric calcium indicator indo-1 AM at 5 ug/ml for 45 minutes and analyzed on the BD LSR II with either a NUVLD (374 nm, 8 mW) or the Nd-YAG (355 nm, 22 mW), using 405/20 and 530/30 nm

filters and a 450 LP splitter. Ionomycin at 2 uM was used to stimulate calcium uptake at 1 minute following initiation of event collection. The NUVLD, due to its longer wavelength, as not applicable for indo-1 analysis; however, the Nd-YAG gave good results

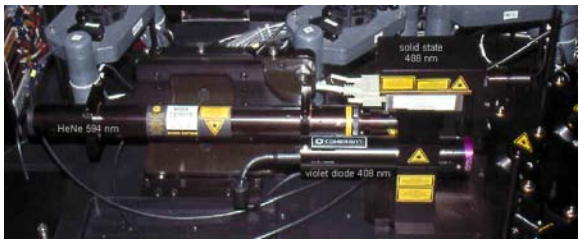
Integration of a HeNe 594 nm yellow laser in the LSR II.

Red helium neon (633 nm) lasers have become important excitation sources for flow cytometry. They efficiently excite a variety of red-excited fluorochromes, including APC and its tandem conjugates APC-Cy5.5 and APC-Cy7, Cy5, Alexa Fluor 633 and 647, the DiI tracer dyes, the dimeric cyanin DNA binding dye TO-PRO-3, and others. HeNe lasers are generally air-cooled, long-lived and simple to operate and maintain. They have largely supplanted the traditional source of orange/red laser excitation, the rhodamine 6G (R6G) dye head laser. Although the RG6 dye head laser provides a valuable range of excitation lines (590 to 620 nm), it has a number of drawbacks; it requires a powerful argon-ion pump laser, employs carcinogenic laser dyes and requires considerable effort to maintain. Red HeNe lasers (generally 20 mW and higher power level) have therefore become common fixtures on cell sorters and laser scanning cytometers.

HeNe lasers with other wavelengths have also been developed; green (543 nm), yellow (594 nm) and orange (612 nm) HeNe lasers are commercially available. HeNe lasers with these longer wavelengths generally cannot exceeded 5 mW in total power output and have not seen extensive use in flow cytometry. HeNe 543 nm lasers (sometimes termed "GreNe" lasers) have been occasionally installed in both small benchtop instruments and large sorters with flow cell capability; the Beckman Coulter Altra can be equipped with small HeNe 543 nm lasers. Nevertheless, longer wavelength HeNe lasers other than 633 nm (specifically 594 and 612 nm) have seen little use in flow cytometry, despite the potential usefulness of their wavelengths; their emission coincides with that of the R6G dye head laser, the optimal excitation source for many red-excited fluorochromes such as Texas Red and APC. HeNe 633 nm lasers cannot excite Texas Red; in addition, the 632 nm line is not spectrally optimal for APC, which has an excitation maxima at 615 nm. Their small size, modest power and cooling requirements and the durability of small HeNe lasers also makes them good candidates as flow cytometry excitation sources.

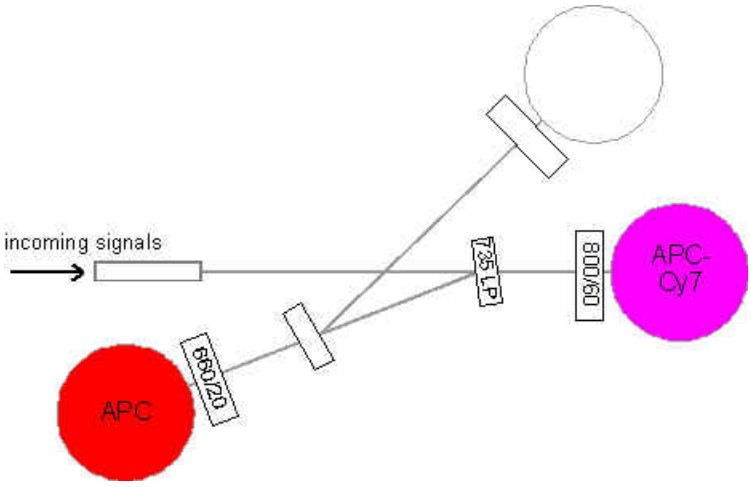
We have previously evaluated NeNe 594 and 612 nm lasers on a FACStar Plus stream-in-air flow cytometer. Although functional, the poor light-collecting efficiency of stream-in-air collection optics limits the usefulness of most low-power laser sources for these instruments. The BD LSR II uses a FACSCalibur-style quartz flow cell and a novel design focusing objective with a numerical aperture of 1.2; the sensitivity of these collection optics make successful integration of a low-power laser a much more feasible prospect. We have therefore integrated our HeNe 594 nm into the LSR II in place of the traditonal red HeNe and used it as an excitation sources for a variety of orange- and red-excited fluorochromes.

(Below). **HeNe 594 nm laser**. We used a stock Melles Griot yellow 594 nm helium-neon laser with maximum measured power output of 3.2 mW. The laser was integrated into the LSR II optical bench in place of the supplied HeNe 633 nm laser and aligned in the fourth laser position..

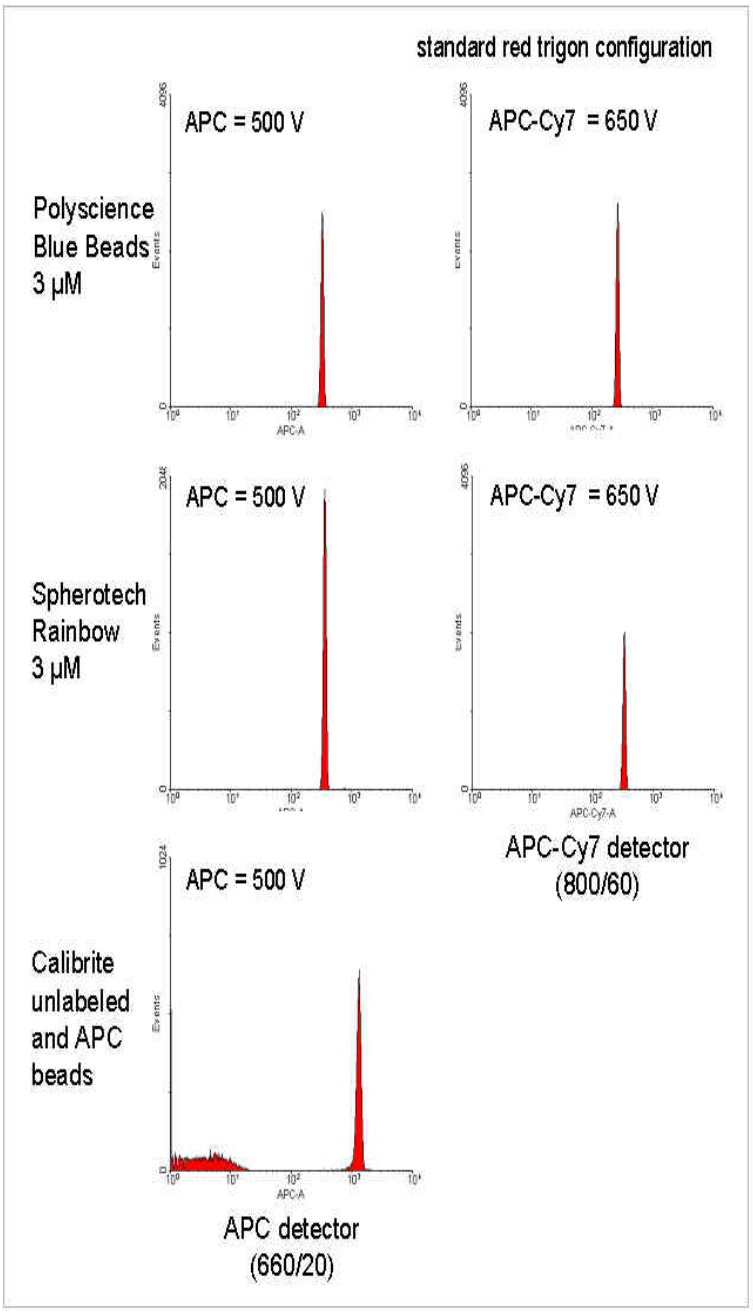


Excitation of APC and APC tandem conjugates with the HeNe 594 nm laser on the LSR II. The yellow HeNe was was first evaluated for its ability to excite APC and its tandem conjugates, a critical requirement for this source.

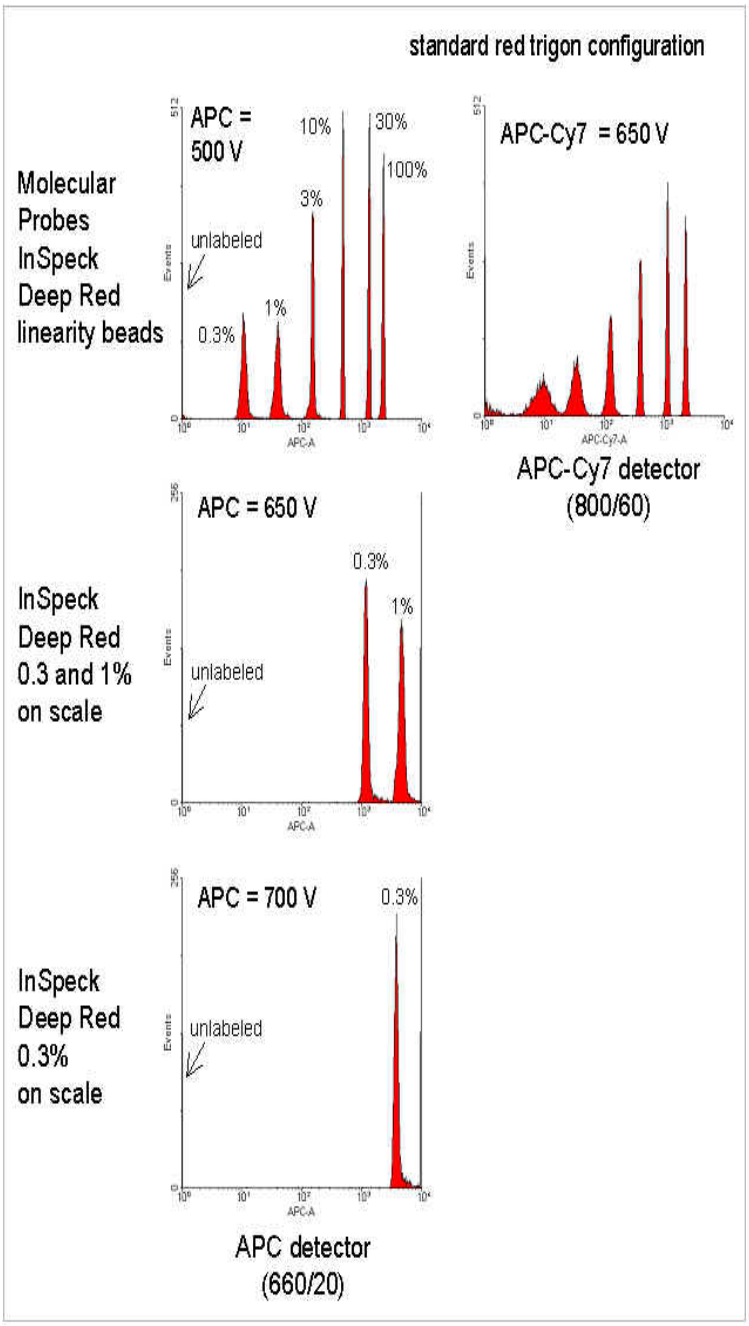
(Below). **HeNe laser trigon configuration.** For this initial evaluation, the LSR II HeNe laser trigon was used in the default configuration, with filters for APC and APC-Cy7 split with a 735 LP dichroic.



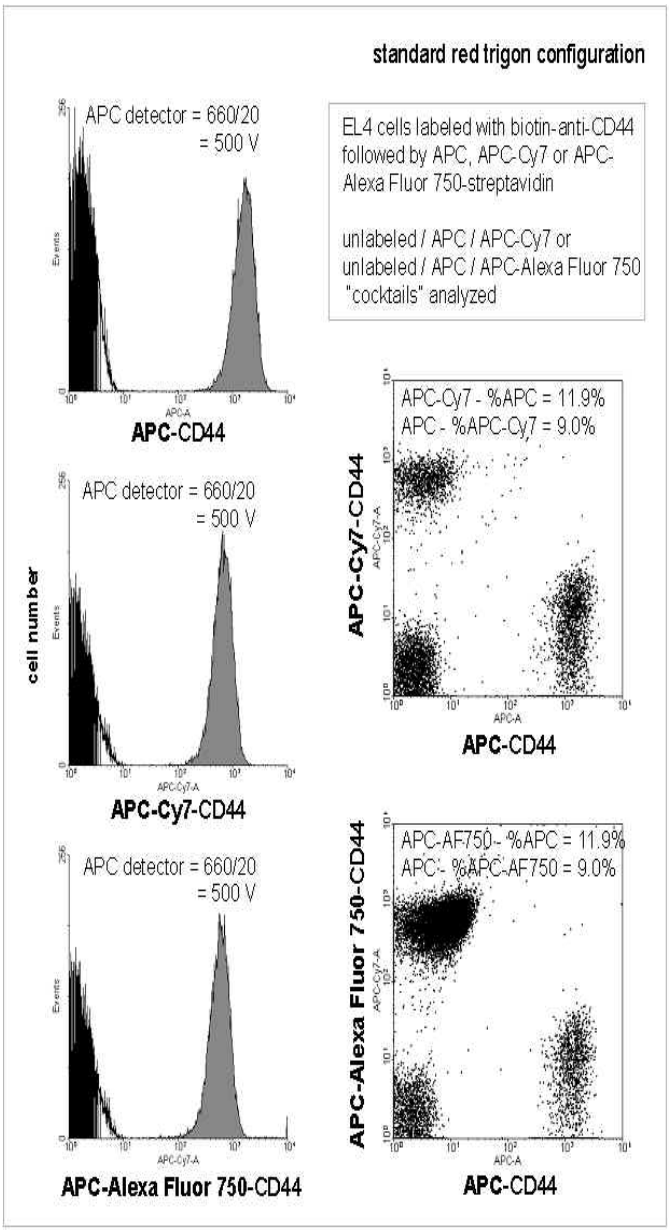
(Below). **Alignment bead analysis.** Polyscience Blue Beads (top histograms), Spherotech rainbow beads (middle) and BD Calibrite APC beads (bottom) were used to align the laser and perform daily quality control evaluations. Bead peak C.V.s and Calibrite unlabeled/APC bead separations were comparable to the normal red HeNe laser.



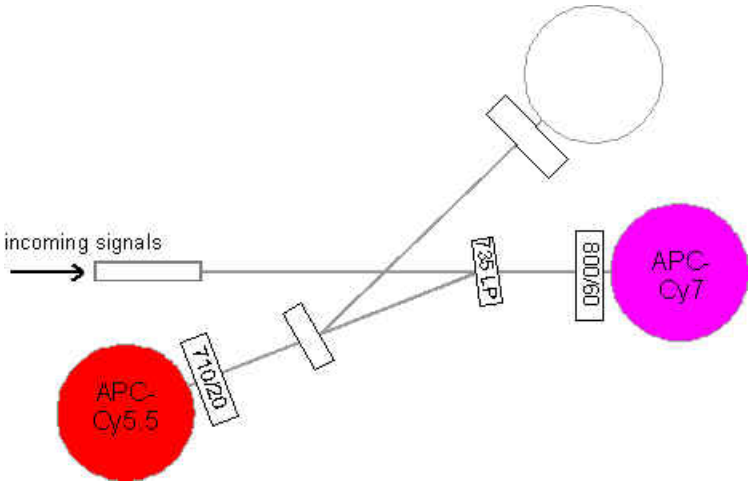
(Below). **Linearity bead analysis with the HeNe 594 nm laser.** Molecular Probes InSpeck Deep Red linearity calibration beads were used to assess detector sensitity using the yellow HeNe laser. Cocktails of beads ranging from an arbitrary 100% fluorescence down to 0.3%, plus unlabeled, were analyzed through both the APC and APC-Cy7 detectors (top histograms). Increasing the detector gain until the 1% or 0.3% bead population was at the scale maximum (middle and bottom hostogram respectively) showed excellent separation between these dim bead populations and the unlabeled fraction.



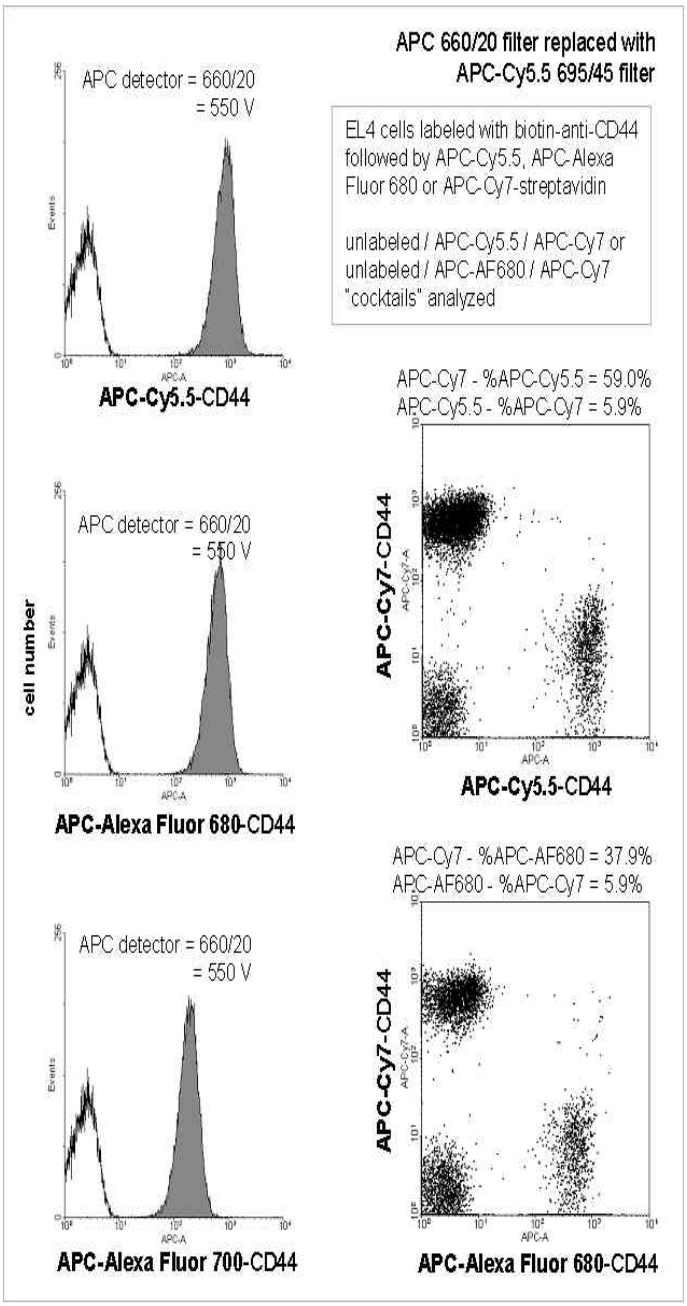
(Below). **Two-color analysis of APC and APC-Cy7 or APC-Alexa Fluor 750 with the HeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC, APC-Cy7 or APC-Alexa Fluor 750-conjugated streptavidin, and "cocktails" of unlabeled, APC, APC-Cy7 or APC-Alexa Fluor 750 labeled cells were analyzed using the yellow HeNe laser. The yellow HeNe gave excellent excitation of both APC and the tandems (left histograms). Two-color analysis of unlabeled / APC / APC-Cy7 or unlabeled / APC / APC-Alexa Fluor 750 "cocktails" (right cytograms) was possible with minimal compensation.



(Below). **Modified HeNe laser trigon configuration.** Evaluation of HeNe 594 nm two-color excitation of APC-Cy5.5 or APC-Alexa Fluor 680 and APC-Cy7 were then carried out using the trigon modification shown below, with a 710/20 nm filter for APC-Cy5.5 or APC-Alexa Fluor 680.

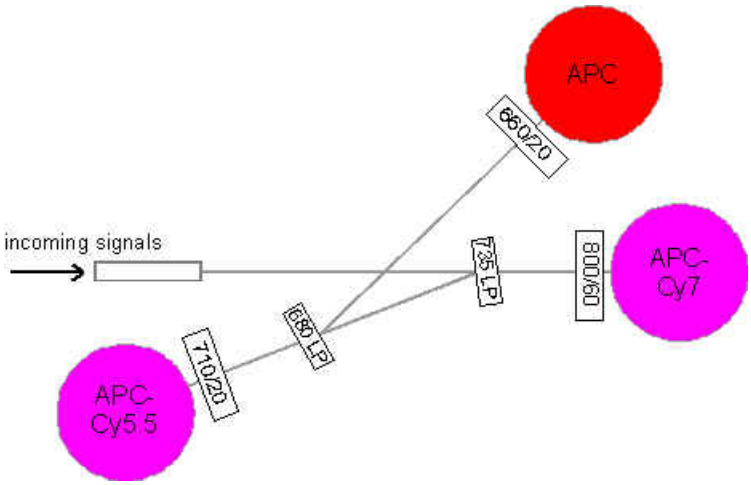


(Below). **Two-color analysis of APC-Cy5.5 or Alexa Fluor 680 and APC-Cy7 with the HeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC-Cy5.5, APC-Alexa Fluor 680 or APC-Cy7 -conjugated streptavidin, and "cocktails" of unlabeled, APC-Cy5.5, APC-Alexa Fluor 680 or APC-Cy7 labeled cells were analyzed using the yellow HeNe laser. Once again, the yellow HeNe gave excellent excitation of all APC tandems (left histograms). Two-color analysis of unlabeled / APC-Cy5.5 / APC-Cy7 or unlabeled / APC-Alexa Fluor 680 / APC-Cy7 "cocktails" (right cytograms) was possible with compensation.

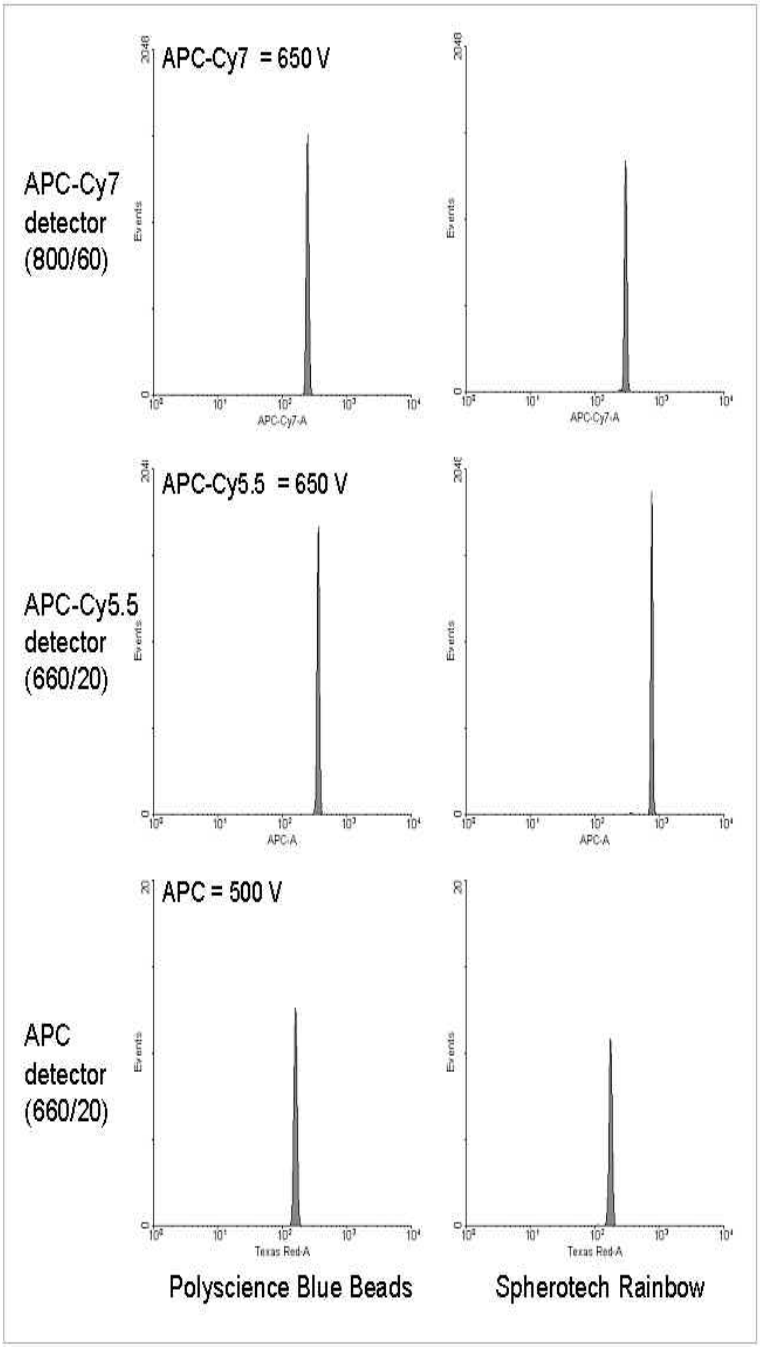


The HeNe 594 nm laser could therefore excite APC and its tandem conjugates in two-color combinations. To determine if APC and two tandems could be simultaneously analyzed in a three-color combination, a third PMT was added to the HeNe trigon and the filters modified for three color analysis.

(Below). **HeNe laser trigon configuration for three-color analysis.** A third PMT was added to the distal detector position of the trigon, and the filters and dichroics modified accordingly.



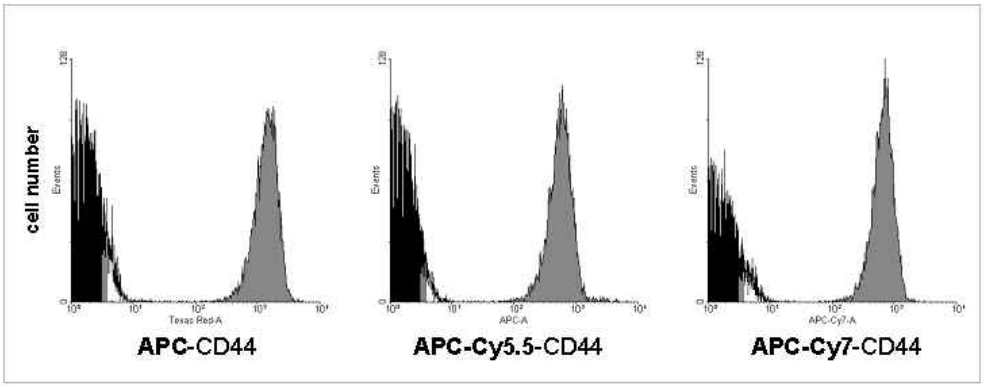
(Below). **Alignment bead analysis.** Polyscience Blue Beads (top histograms) and Spherotech rainbow beads (bottom) were used to align the laser and perform daily quality control evaluations. Bead peak C.V.s were comparable to the normal red HeNe laser.

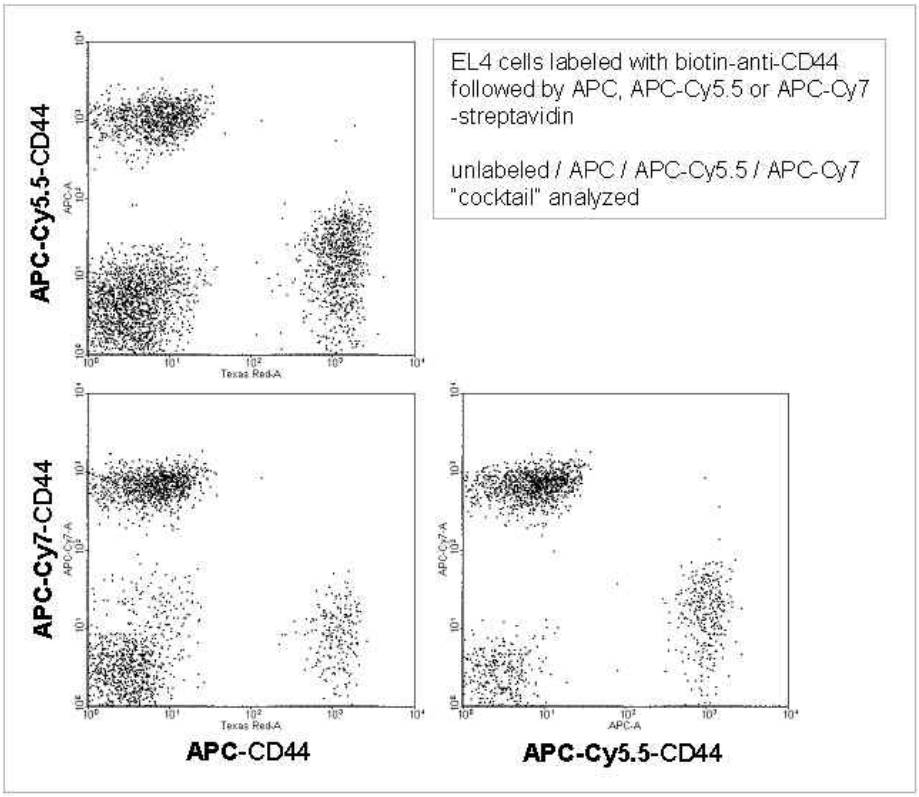


(Below). **Linearity bead analysis with the HeNe 594 nm laser.** Molecular Probes InSpeck Deep Red linearity calibration beads were used to assess detector sensitity using the yellow HeNe laser. Cocktails of beads ranging from an arbitrary 100% fluorescence down to 0.3%, plus unlabeled, were analyzed through both the APC-Cy7, APC-Cy5.5 and APC detectors (top to bottom).

(Below). **Three-color analysis of APC, APC-Cy5.5 and APC-Cy7 with the HeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC, APC-Cy5.5 or APC-Cy7-conjugated streptavidin, and "cocktails" of unlabeled, APC, APC-Cy5.5 and APC-Cy7 labeled cells were analyzed

using the yellow HeNe laser. The yellow HeNe still gave excellent excitation of both APC and the tandems (top histrograms). Three-color analysis of unlabeled / APC / APC-Cy5.5 / APC-Cy7 "cocktails" (lower three cytograms) was possible with compensation.

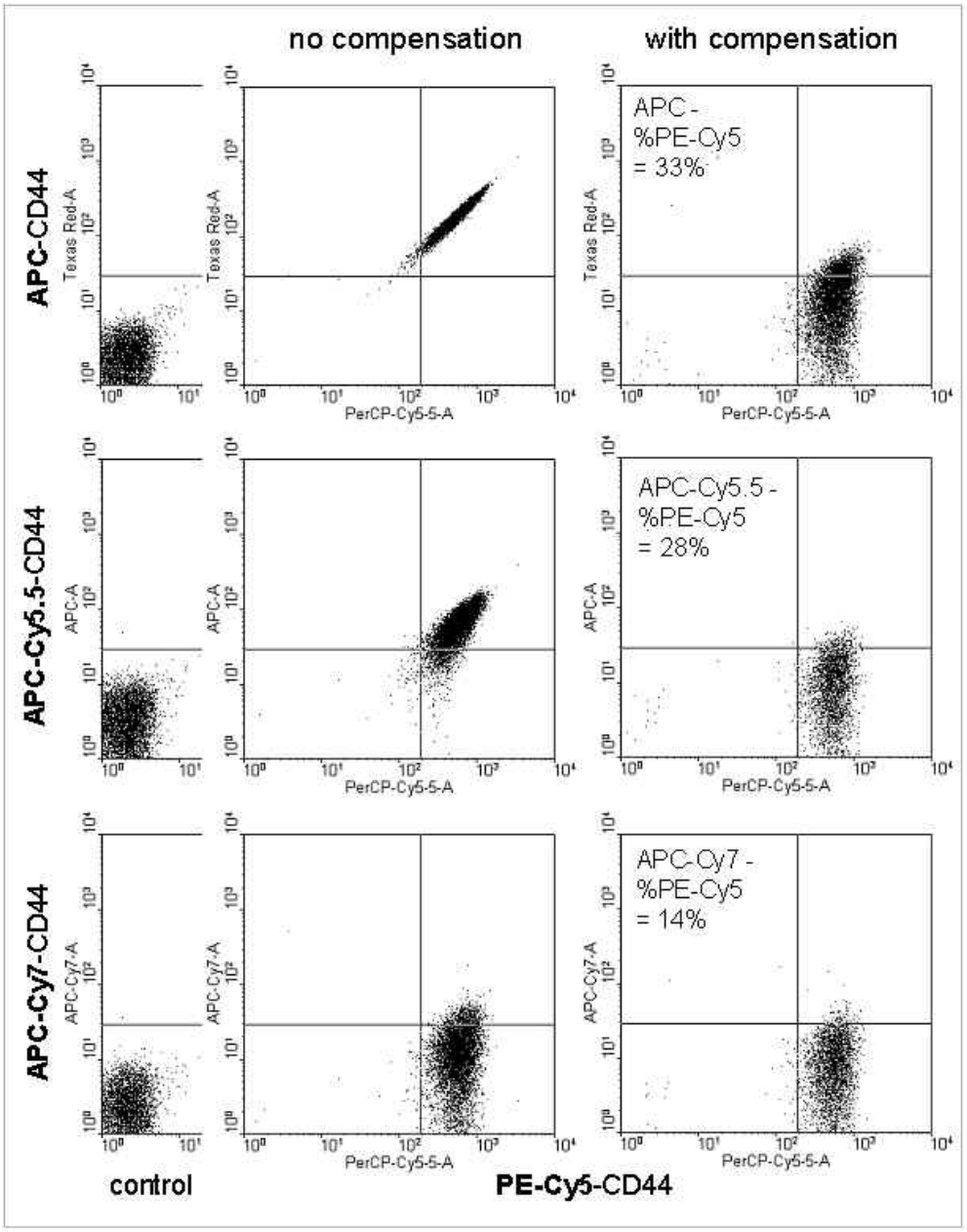




An important problem with using red-excited flurochromes is spectral interference by the fluorochrome PE-Cy5, the phycoerythrin Cy5 tandem conjugate. Since Cy5 is excited by red laser sources such as the red HeNe, its coincidental emission with APC usually requires a large level of color compensation to control spectral bleedover and allow simultaneous use of PE-Cy5 and APC. Since the yellow HeNe emits a shorter wavelength than is optimal for Cy5 excitation, it was expected that APC / PE-Cy5 crossbeam compensation problem should be reduced using this laser. PE-Cy5 bleedover and the resulting crossbeam compensation requirement was therefore evaluated for the

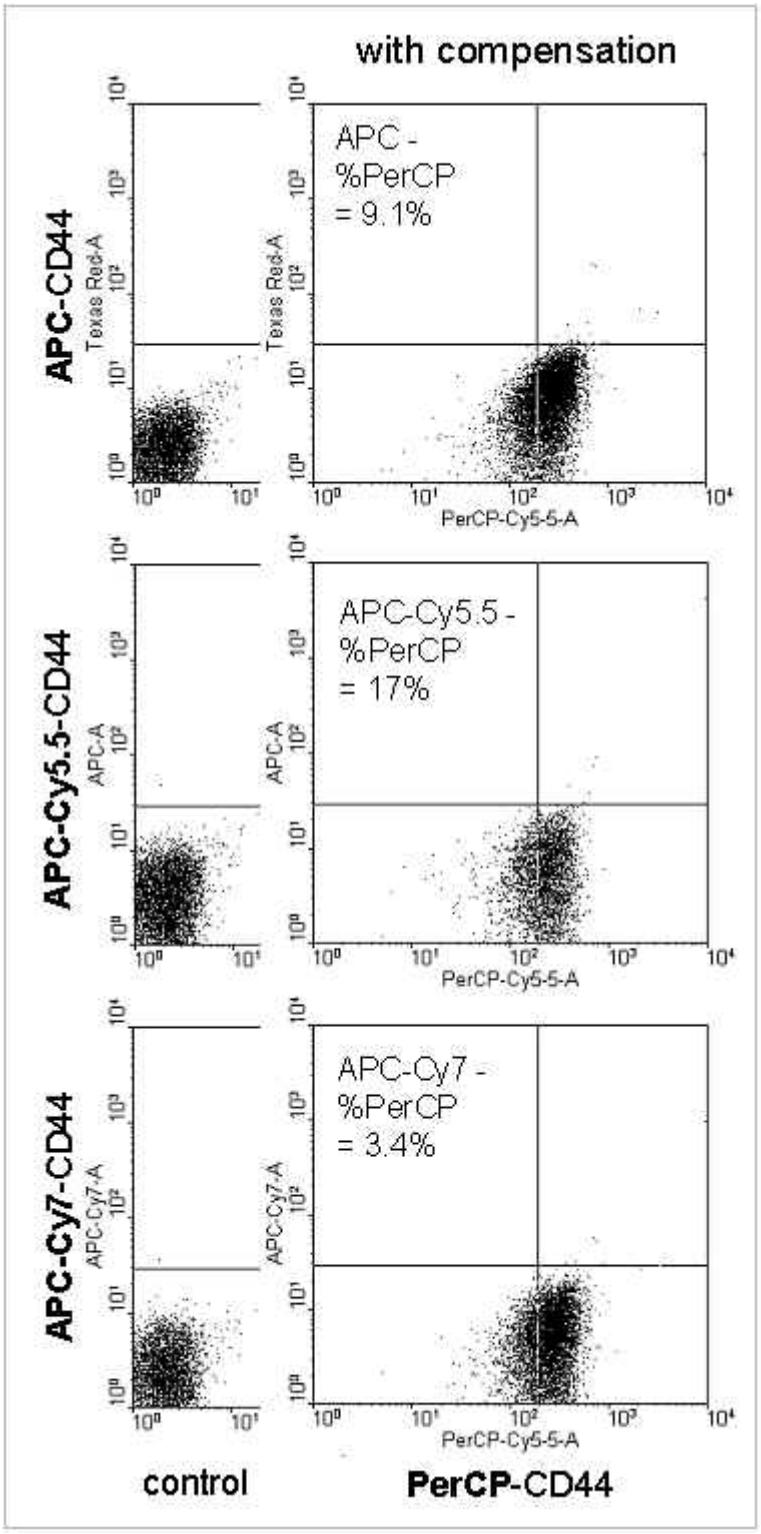
APC and APC tandem detectors. Spectral overlap of the fluorochrome PerCP into the APC and APC tandem detectors was similarly evaluated.

(Below). **Crossbeam compensation between PE-Cy5 and APC and its tandems.** EL4 cells were labeled with biotin-conjugated CD44 followed by PE-Cy5-conjugated streptavidin, and analyzed using the yellow HeNe laser, observing the spectral overlap into the APC and APC tandem detectors. The crossbeam compensation was then adjusted to correct for spectral overlap, and the value noted below. Overlap into the APC (top cytograms), APC-Cy5.5 (middle) and APC-Cy7 (below) are shown, both without (left column) and with compensation (right column).



(Below). **Crossbeam compensation between PE-Cy5 and APC and its tandems.** As above, EL4 cells were labeled with biotin-conjugated CD44 followed by PerCP-conjugated streptavidin, and analyzed using the yellow HeNe laser, observing the spectral overlap into the APC and APC tandem detectors. The

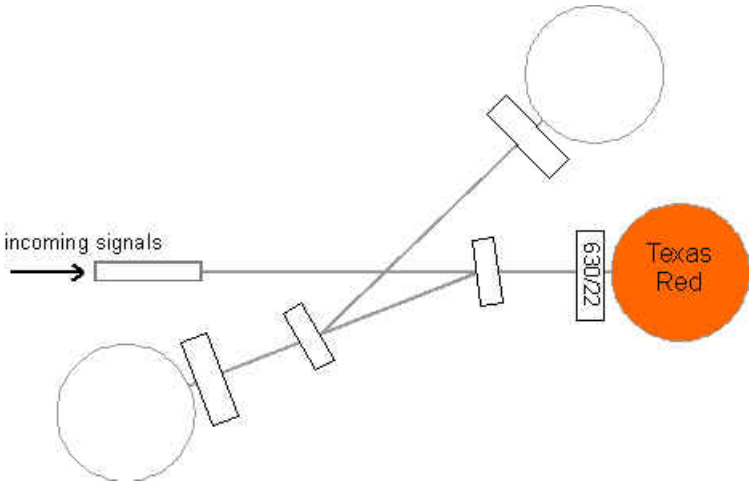
crossbeam compensation was then adjusted to correct for spectral overlap, and the value noted below. Overlap into the APC (top cytograms), APC-Cy5.5 (middle) and APC-Cy7 (below) are shown.



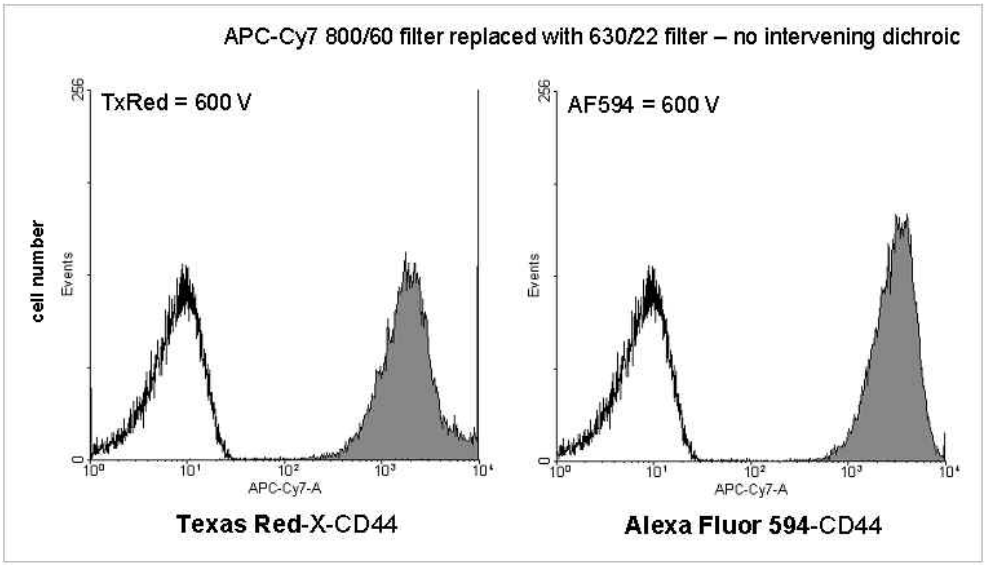
Crossbeam compensation requirements for PE-Cy5 and APC and its tandems using the yellow HeNe were at acceptable levels. As expected, the crossbeam compensation requirements for PerCP were much lower. Comparisons to a red HeNe source are in progress.

Excitation of Texas Red and Alexa Fluor 594 with the HeNe 594 nm laser on the LSR II. The main advantage of the yellow HeNe laser for flow cytometer is the ability to excite Texas Red and its more recent analog Alexa Fluor 594, useful low molecular weight fluorochromes that cannot be excited with red HeNe sources. Texas Red or Alexa Fluor 594 can theoretically be combined with APC and it tandems to do four-color analysis off one yellow HeNe laser, a significant advantage when doing polychromatic flow cytometer (seven colors and beyond).

(Below). **HeNe laser trigon configuration for Texas Red or Alexa Fluor 594 detection.** To initially evaluate the ability of the yellow HeNe to excite Texas Red and Alexa Fluor 594, all dichroics were removed from the HeNe trigon, and a 630/22 nm filter inserted in front of the proximal detector. Although a 610 nm would be more optimal from a detection perspective, the spectral proximity to the 594 nm line resulted in high backgrounds and necessitated a longer wavelength filter.

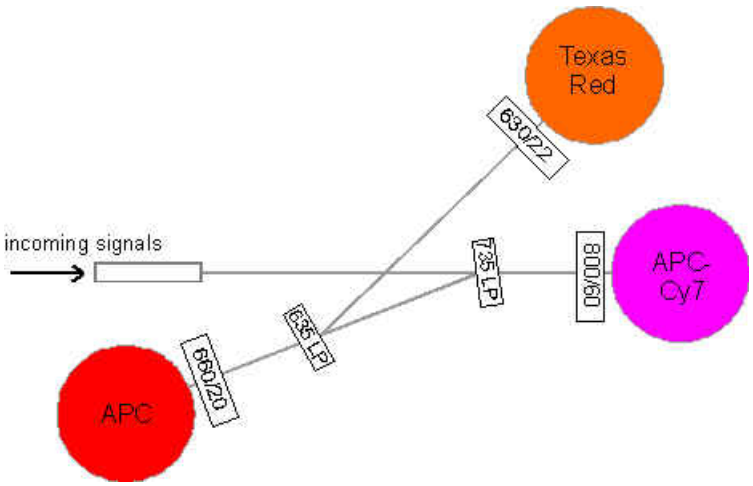


(Below). **Detection of Texas Red and Alexa Fluor 594 on the LSR II with the HeNe 594 nm laser.** The yellow HeNe gave excellent excitation of both Texas Red and Alexa Fluor 594.

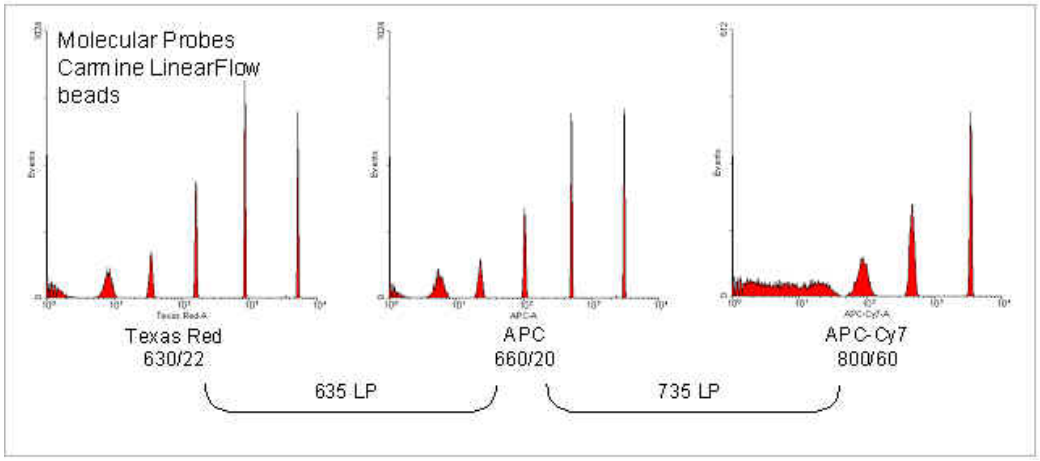


Combining Texas Red or Alexa Fluor 594 with APC and its tandems for three- and four-color analysis with the HeNe 594 nm laser on the LSR II. The ability of the yellow HeNe to excite Texas Red and Alexa Fluor 594 opens the possibility of three- and four-color analysis with APC and its tandems. The HeNe trigon is limited to three-color analysis using the configurations shown below. The yellow laser was found to excite Texas Red or Alexa Fluor 594, APC and APC-Cy7 simultaneously with good signal separation and reasonable compensation.

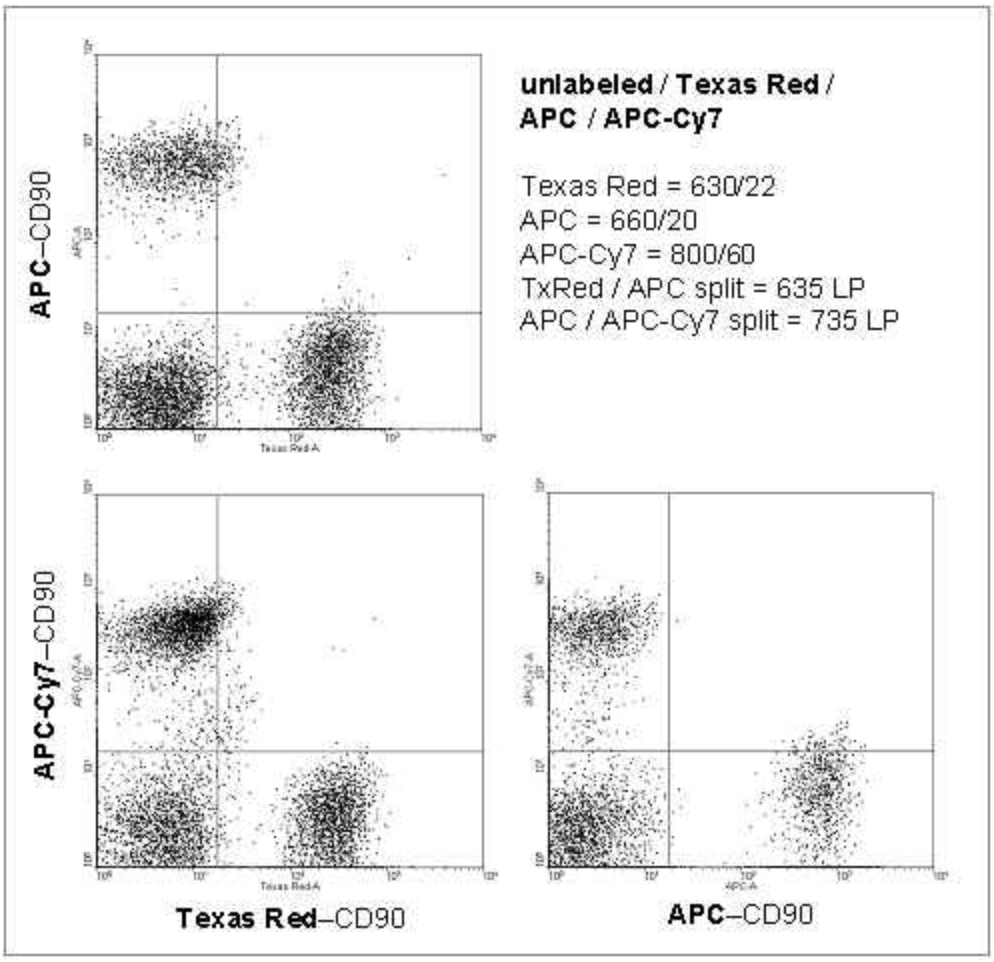
(Below). **HeNe laser trigon configuration for Texas Red or Alexa Fluor 594 detection with APC and APC-Cy7.** This configuration requires a custom 635 LP dichroic.

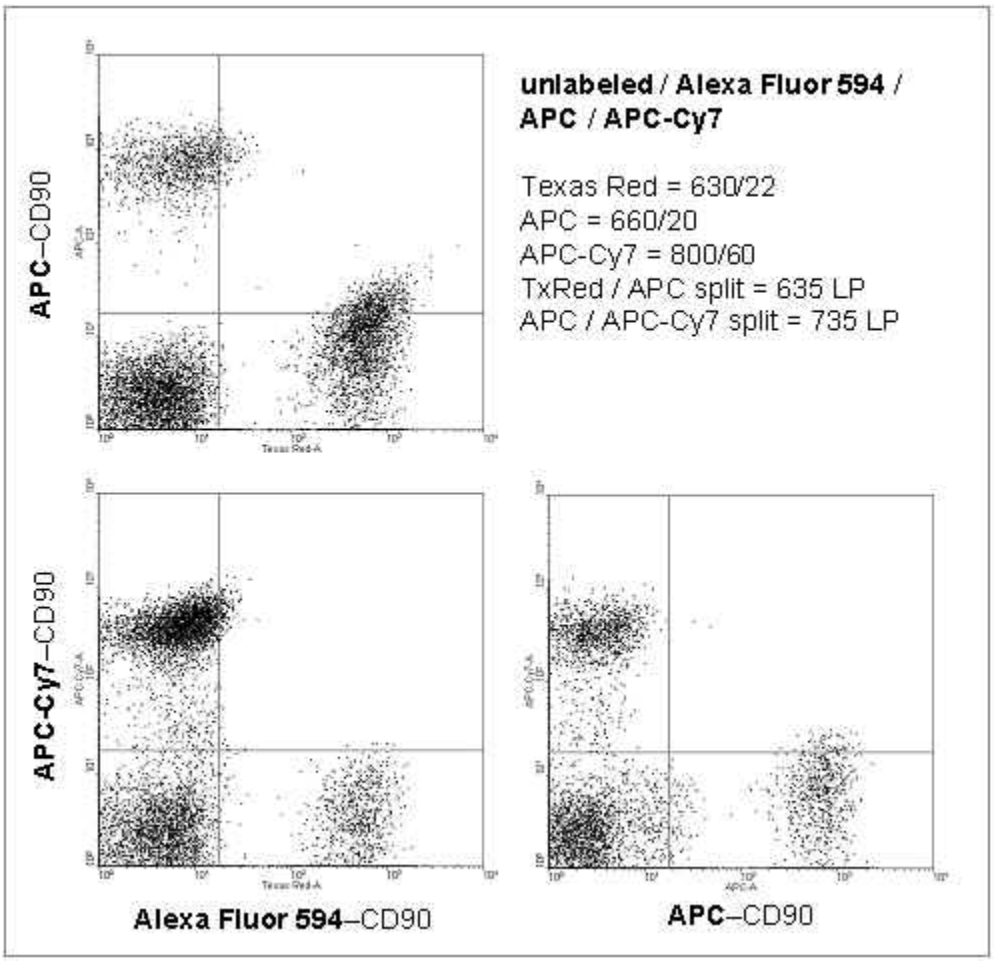


(Below). **Linearity bead analysis with the HeNe 594 nm laser.** Molecular Probes Carmine LinearFlow linearity calibration beads were used to assess detector sensitity using the yellow HeNe laser. Cocktails of beads ranging from an arbitrary 100% fluorescence down to 0.02% were analyzed through both the Texas Red, APC and APC-Cy7 detectors.



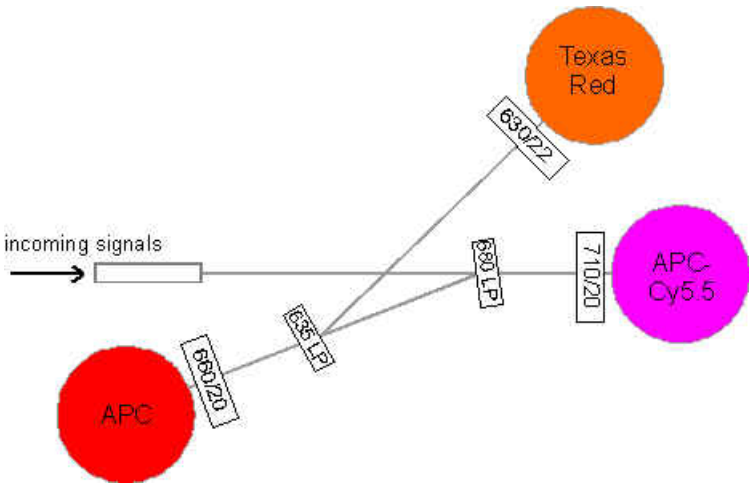
(Below). **Three-color analysis of Texas Red or Alexa Fluor 594, APC and APC-Cy7 using the HeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by Texas Red, Alexa Fluor 594, APC or APC-Cy7-conjugated streptavidin, and "cocktails" of unlabeled, Texas Red, APC and APC-Cy7 (left panels) or Alexa Fluor 594, APC and APC-Cy7 (right panels) labeled cells were analyzed using the yellow HeNe laser. A customized 630 LP dichroic was used to split the Texas Red or Alexa Fluor 594 and APC signals.


unlabeled / Texas Red / APC / APC-Cy7
Texas Red = 630/22
APC = 660/20
APC-Cy7 = 800/60
TxRed / APC split = 635 LP
APC / APC-Cy7 split = 735 LP
APC–CD90
APC-Cy7–CD90
Texas Red–CD90
APC–CD90

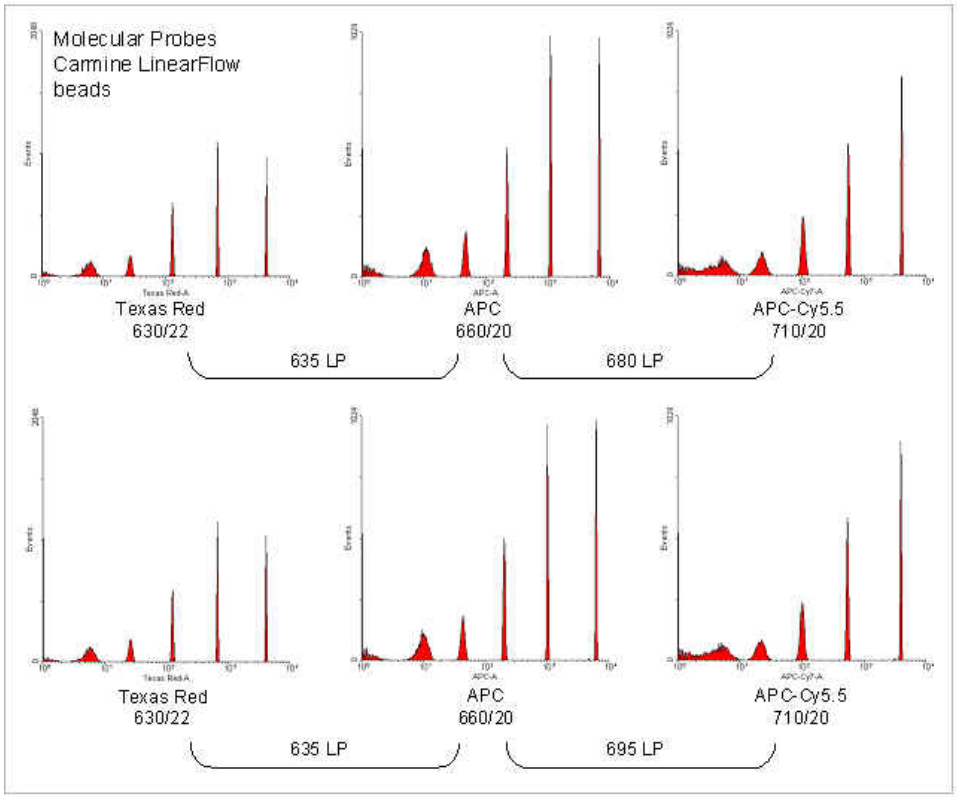


The yellow laser was also able to excite Texas Red, APC and APC-Cy5.5 simultaneously, also with reaonable compensation levels. This suggests that excitation of four fluorochromes (Texas Red, APC, APC-Cy5.5 and APC-Cy7) should be possible with the replacement of the current trigon with an octagon.

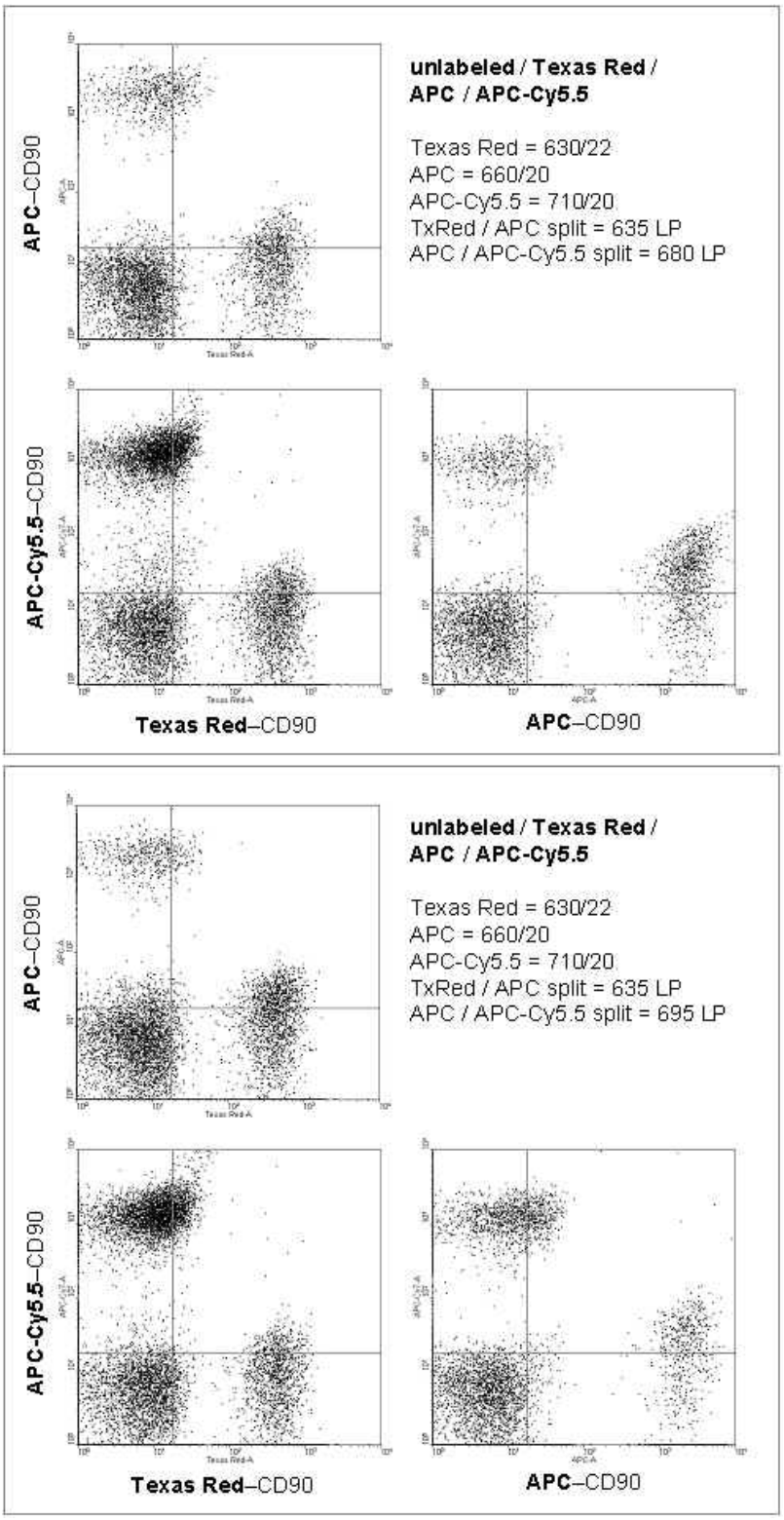
(Below). **HeNe laser trigon configuration for Texas Red or Alexa Fluor 594 detection with APC and APC-Cy5.5.** Analysis was done with the default 680 LP dichroic shown, or with a custom 695 LO dichroic.



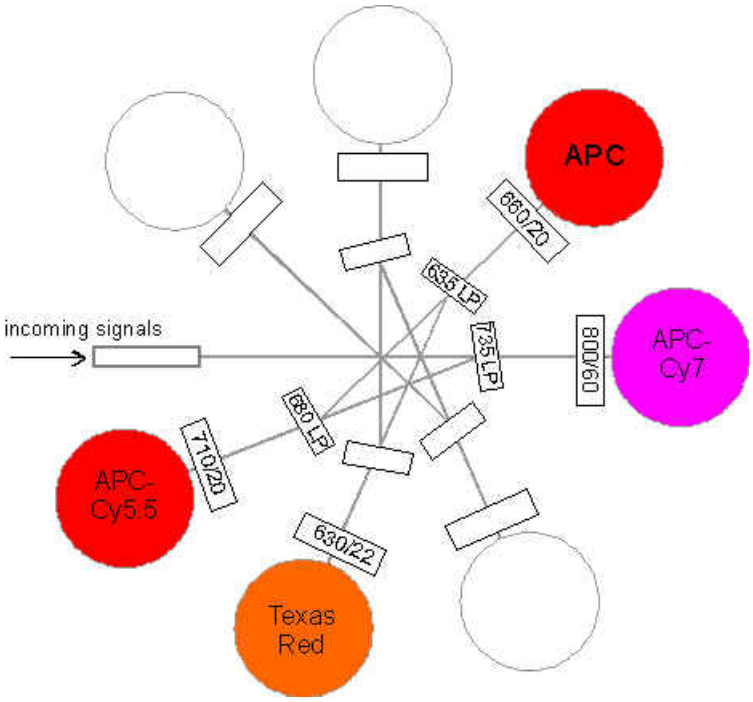
(Below). **Linearity bead analysis with the HeNe 594 nm laser.** Molecular Probes Carmine LinearFlow linearity calibration beads were used to assess detector sensitity using the yellow HeNe laser. Cocktails of beads ranging from an arbitrary 100% fluorescence down to 0.02% were analyzed through both the Texas Red, APC and APC-Cy5.5 detectors. 680 LP (upper row) or 695 LP (lower row) dichroics were used to separate the APC and APC-Cy5.5 signals.



(Below). **Three-color analysis of Texas Red or Alexa Fluor 594, APC and APC-Cy5.5 using the HeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by Texas Red, APC or APC-Cy5.5-conjugated streptavidin, and "cocktails" of unlabeled, Texas Red, APC and APC-Cy.5 labeled cells were analyzed using the yellow HeNe laser. 680 LP (left panels) or 695 LP (right panels) dichroics were used to separate the APC and APC-Cy5.5 signals.



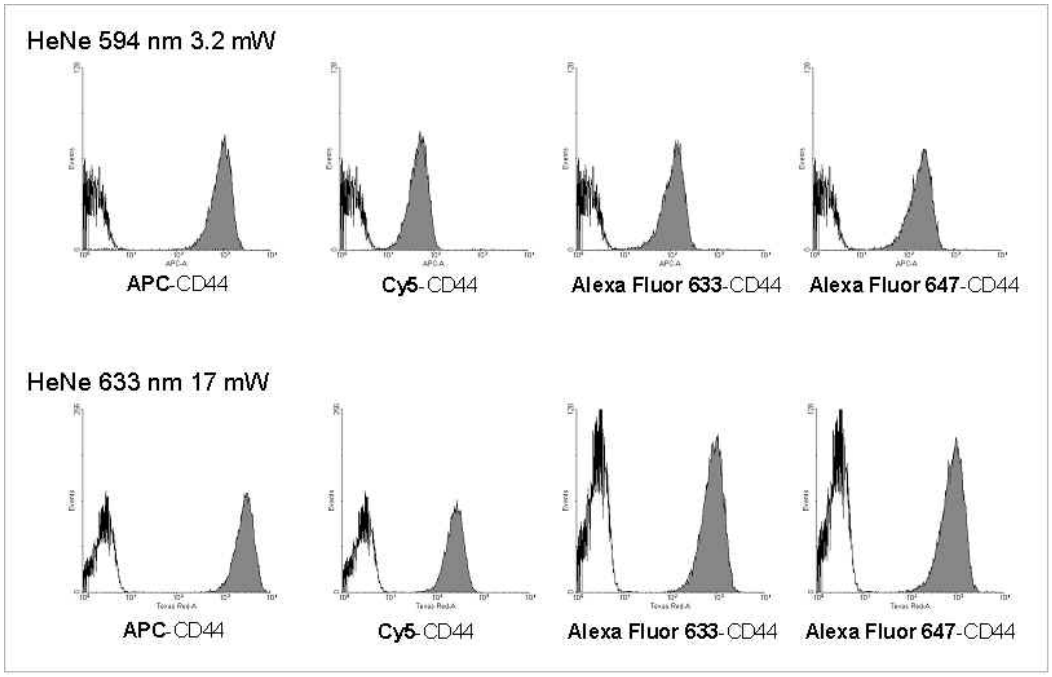
(Below). **Proposed HeNe laser octagon configuration for Texas Red or Alexa Fluor 594 detection with APC, APC-Cy5.5 and APC-Cy7.** We are planning to substitute a PMT octagon for the existing trigon, for four-color analysis with the yellow HeNe.



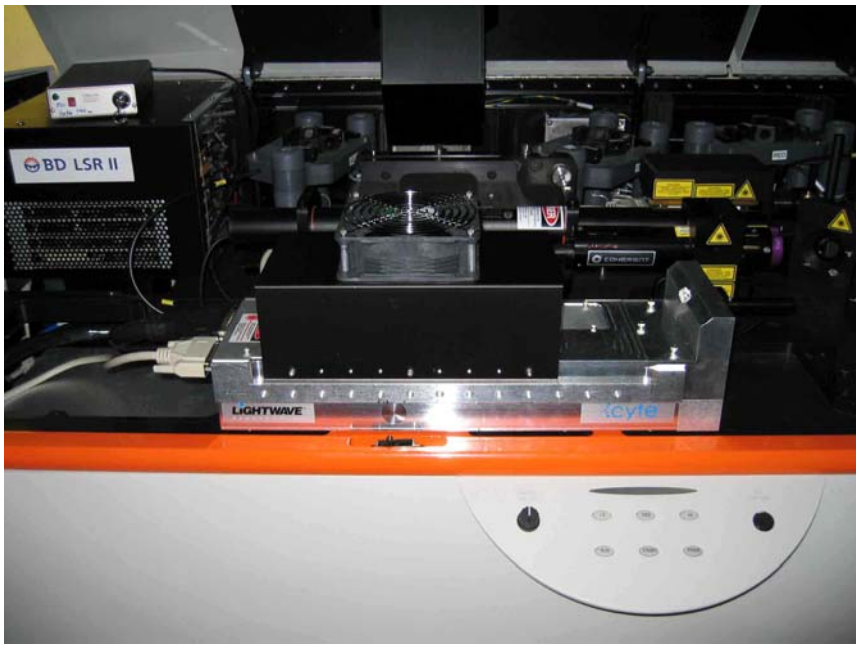
Configuration of this design is in progress.

Excitation of low molecular weight red-excited fluorochromes with the HeNe 594 nm laser on the LSR II. One possible drawback of the yellow HeNe laser is its reduced ability to excite several low molecular weight red-excited fluorochromes, including Cy5 and Alexa Fluor 633 and Alexa Fluor 647. The results show some reduction in sensitivity for these probes compared to the red HeNe, which should be taken into account in both flurochrome and laser choice.

(Below). **Cy5, Alexa Fluor 633 and Alexa Fluor 647 excitation with the NeNe 594 nm laser.** EL4 cells were labeled with biotin-conjugated CD44 followed by APC, Cy5, Alexa Fluor 633 or Alexa Fluior 647 conjugated streptavidin, and analyzed as "cocktails" with unlabeled cells with either the HeNe 594 laser (top histograms) or the HeNe 632 nm (bottom).



Below). **Lightwave Electronics Nd-YAG UV laser, final installation.** The BD install of the Nd-YAG is shown below. Since there is minimal beam alignment possible with the factory install, the flow cell mest be aligned to the UV beam, and all other lasers subsequently aligned to the flow cell.


BD LSR II
LIGHTWAVE
cyte