

Solid State Yellow and Orange Lasers for Flow Cytometry

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• Abstract

Diode and DPSS lasers emitting a variety of wavelengths are now commonly incorporated into flow cytometers, greatly increasing our capacity to excite a wide variety of fluorochromes. Until recently, however, virtually no practical technology existed for generating yellow or orange laser light for flow cytometry that was compatible with smaller instrumentation. In this study, we evaluate several new solid state laser systems that emit from the 570 to 600 nm as excitation sources for flow cytometry. DPSS 580, 589, and 592 nm sources were integrated into a cuvette-based flow cytometer (BD LSR II) and a stream-in-air cell sorter (FACSVantage DiVa), and used to excite a variety of yellow, orange, and red excited fluorochromes, including Texas Red, APC, and its tandem conjugates, and the genetically encoded red fluorescent protein HcRed and the more recently developed Katushka. All laser sources were successfully incorporated into the indicated flow cytometry platforms. The yellow and orange sources (particularly 592 nm) were ideal for exciting Texas Red, and provided excitation of APC and its tandems that was comparable to a traditional red laser source, albeit at higher power levels than red sources. Yellow and orange laser light was optimal for exciting HcRed and Katushka. Practical yellow and orange laser sources are now available for flow cytometry. This technology fills an important gap in the laser wavelengths available for flow, now almost any fluorochrome requiring visible light excitation can be accommodated. Published 2008 Wiley-Liss, Inc.†

• Key terms

yellow laser; orange laser; diode pumped solid state; red fluorescent protein; Texas Red; allophycocyanin; HcRed; Katushka

SOLID state laser technology (diode and diode-pumped solid state, or DPSS) has recently produced a variety of novel laser sources that have proven useful for flow and image cytometry (1). These lasers are small and durable enough for integration into small benchtop cuvette-based instrumentation, and often possess sufficient power levels to accommodate stream-in-air cell sorting (2). Ultraviolet (370–380 nm), violet (395–415 nm), and blue (435–445 nm) laser diodes have all been incorporated into flow cytometers and are being used for exciting a wide range of useful fluorescent probes (3–6). DPSS lasers are now available in the blue–green range (473–515 nm), with the DPSS 488 nm variant replacing traditional ion lasers for many instrument applications. Green (532 nm) and green–yellow (561 nm) DPSS lasers have proven useful for efficient excitation of phycoerythrin and its tandems, as well as rhodamine-based fluorochromes and GFP-like long-wavelength fluorescent proteins (2,7,8). These lasers are also starting to appear on commercial flow cytometry instrumentation, vastly improving our ability to utilize the full spectrum of fluorescent probes available for biomedical analysis.

However, until very recently, a significant gap existed in the range of laser wavelengths available for flow cytometry. The yellow to orange bandwidth (~570–620 nm) was very difficult to achieve using existing technology. Yellow and orange

laser light could be generated using a dye head laser charged with a laser dye such as rhodamine 6G; however, dye head lasers require extremely powerful (~5 W) argon or krypton ion lasers to pump the laser dye. The resulting systems were large, maintenance-intensive and expensive and could only be accommodated on large-frame cell sorters. Helium-neon 594 and 612 nm lines could be generated, but at efficiencies far lower than for the familiar red 633 nm line. Yellow and orange HeNe lasers have maximum power levels of 5 mW and often significantly less. Although these lasers have been useful for confocal microscopy applications, their low power levels have limited their use in flow cytometry.

This gap has been unfortunate, because yellow to orange excitation would be a very useful tool for flow cytometry. APC has been previously demonstrated to be well excited by HeNe 594 nm laser light, despite its low power; it is expected that excitation should be nearly as effective as with traditional red laser sources (1). Yellow and orange laser sources would also be ideal for exciting Texas Red and its photostable analog Alexa Fluor 594. These low molecular weight fluorescent probes are very bright and photostable, but have seen little use in flow cytometry due to the lack of a good excitation source. Texas Red, APC, and the APC tandems can also be combined for multicolor analysis when an orange laser source is used for excitation. Finally, many of the recently cloned red fluorescent proteins (RFPs), including HcRed, mPlum, mKate, and Katushka have excitation maxima that exceed 580 nm; these fluorescent proteins would optimally require an excitation source in the yellow to orange range (9–11). These probes have been used extensively for imaging, but have seen only limited use in flow cytometry.

Very recent advances in laser technology have finally produced solid state yellow and orange laser sources that are practical for integration into commercial flow cytometers. In this study, we have evaluated several such sources; all are continuous wave (CW) sources, and all are based on DPSS technology. Two of them (a 580 and a 592 nm source) are fiber lasers, where a specially doped fiber optic constitutes the lasing cavity (12). A third 589 nm free space laser uses a traditional solid state lasing cavity. All possess power levels, beam characteristics, and low noise levels sufficient for use in flow cytometry. All of these lasers were tested for excitation of a variety of yellow, orange, and red excited fluorochromes, in comparison with existing shorter or longer wavelength lasers where applicable.

MATERIALS AND METHODS

Cells and Immunophenotyping

EL4 cells were obtained from the ATCC (Manassas, VA) and maintained in RPMI containing 10% FBS. Cells were labeled with biotin-conjugated anti-mouse CD44 primary antibody, and secondary labeled with streptavidin conjugated to Texas Red, Alexa Fluor 594, Alexa Fluor 647, allophycocyanin (APC), APC-Cy5.5, or APC-Cy7. All immunolabeling reagents were obtained from Invitrogen Corporation (Carlsbad, CA).

Bacteria and Fluorescent Protein Expression

The genes encoding HcRed or Katushka were inserted into pBAD/His-B vector (Invitrogen) using *Bgl*III and *Eco*RI

restriction sites. The resulting pBAD-HcRed or pBAD-Katushka plasmids were transformed into electrocompetent *E.coli* bacterial strain LMG194 (Invitrogen). The protein expression was then induced with 0.02% (wt/vol) L-arabinose at 37°C. For flow cytometry, bacterial cells expressing the proteins were washed with phosphate-based saline (PBS), fixed with 4% paraformaldehyde and resuspended in PBS with OD = 0.01 measured at 650 nm.

Lasers and Flow Cytometry

The following green–yellow, yellow, and orange laser sources were evaluated:

1. DPSS 558 nm, 20 mW fixed power level (NEL America, Saddle Brook, NJ).
2. DPSS 561 nm, 50 mW fixed power level (Cobolt, Stockholm, Sweden).
3. DPSS 580 nm fiber laser, 200 mW maximum power level (MPB Communications, Inc., Montreal, Quebec, Canada).
4. DPSS 589, 20 mW fixed power level (NEL America, Saddle Brook, NJ).
5. DPSS 592 nm fiber laser, 200 mW maximum power level (MPB Communications).

The DPSS 558 and 589 nm lasers were nonadjustable and used at a fixed power level. The DPSS 561 nm laser also operated at a fixed level of 50 mW but could be attenuated with a neutral density filter down to 20 mW. The power level of the DPSS 580 and 592 nm fiber lasers could be varied down to 50 mW while maintaining low noise levels and were used at the level indicated in the figures. For DPSS 580 nm laser power levels below 50 mW, a neutral density filter was also used to attenuate the power level while maintaining a low noise level. Comparisons were sometimes made to a HeNe 633 nm laser emitting at ~20 mW (JDS Uniphase). All laser power levels were monitored using a power meter with semiconductor detector head (Thorlabs, Newton, NJ). All indicated lasers were mounted on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). The DPSS 592 nm fiber laser was also mounted on a stream-in-air FACS Vantage DiVa cell sorter (BD Biosciences). Laser beam shape was modified using a 3× beam expander (Melles Griot), and beam profiles were measured using a DataRay CCD camera (DataRay, Boulder Creek, CA). The resulting beam profiles for all evaluated lasers were normalized to a Gaussian distribution with a 50% beam waist diameter of ~0.8 mm at a distance immediately prior to the LSR II laser focusing lens. Instrument alignment was optimized using Spherotech Ultra Rainbow Fluorescent Particles single intensity microspheres (catalog number URFP-38-2) and sensitivity for each laser sources assessed using Spherotech Rainbow Calibration Particles 8-population microsphere mixtures (catalog number RCP-30-5A) (Spherotech, Lake Forest, IL). Alignments and microspheres assessments were usually made using a 660/20 nm bandpass filter. Texas Red and Alexa Fluor were detected through a 630/22 nm bandpass filter, Alexa Fluor 647, APC and HcRed and Katushka through a 660/20 nm, APC-Cy5.5 through a 710/50 nm, and APC-Cy7

TECHNICAL NOTE

through an 800/60 nm. PMT voltages were fixed for each laser power level; PMT voltages were increased by 20 V in the 20 mW laser tests compared to 50 mW. Data was acquired using the DiVa acquisition software system (BD Biosciences), exported as FCS 3.0 files and analyzed using FlowJo for PC ver. 7.2.4 on a five log scale (TreeStar Software, Ashland, OR). Data was initially expressed as median fluorescence intensity (MFI) values for both the labeled and unlabeled cell populations (henceforth designated Signal and Background, respectively). This raw data is shown in Supplementary Table 1. The following sensitivity metrics were then used to estimate signal detection over background (13,14):

1. $(\text{Signal media} - \text{Background median}) / (\text{robust SD})$. The robust SD is estimated as the (84 percentile of the background median minus the background median, divided by 0.995. This metric is termed a sensitivity index or staining index (SI) and accounts for error and peak spreading in the background fluorescence distribution. This metric is displayed on the histograms, and is designated SI-1.
2. $(\text{Signal media} - \text{Background median}) / (95 \text{ percentile of background median} - \text{background median})$. This metric is somewhat less sensitive to background data with low values, and is designated SI-2. These values are included in Supplementary Table 1.
3. $(\text{Signal media} - \text{Background median}) / (95 \text{ percentile of background median} - \text{background median}) + (\text{Signal median} - 5 \text{ percentile of Signal median})$. This method attempts to give more weight to the distribution and spread of the labeled population, and is designated SI-3. These values are also included in Supplementary Table 1.

RESULTS

Several novel yellow and orange laser sources were evaluated for their ability to excite a variety of phenotyping fluorochromes and a representative red fluorescent protein by flow cytometry. Fiber 580 and 592 nm sources and free-space 558 and 589 nm modules are shown in Figure 1. Where applicable, these lasers were tested concurrently with shorter-wavelength green–yellow lasers (558 and 561 nm), as well as a longer wavelength red HeNe. Alignment verification and sensitivity assessments are shown in Figure 2, where the indicated lasers were mounted on a cuvette-based BD LSR II and used to analyze Spherotech Rainbow eight-population microspheres. The lasers were compared at similar power levels, including a series ranging from 558 to 633 nm and running at 20 mW (left column), and a series ranging from 561 to 592 nm and running at 50 mW. All lasers could be easily integrated into the cytometer with good beam shape and alignment, and all were able to resolve all eight Spherotech populations. Although this data does not indicate the applicability of these lasers for any particular fluorochrome (since Spherotech Rainbow beads incorporate multiple fluorochromes of proprietary identity), it does indicate consistent and comparable alignment between laser types.

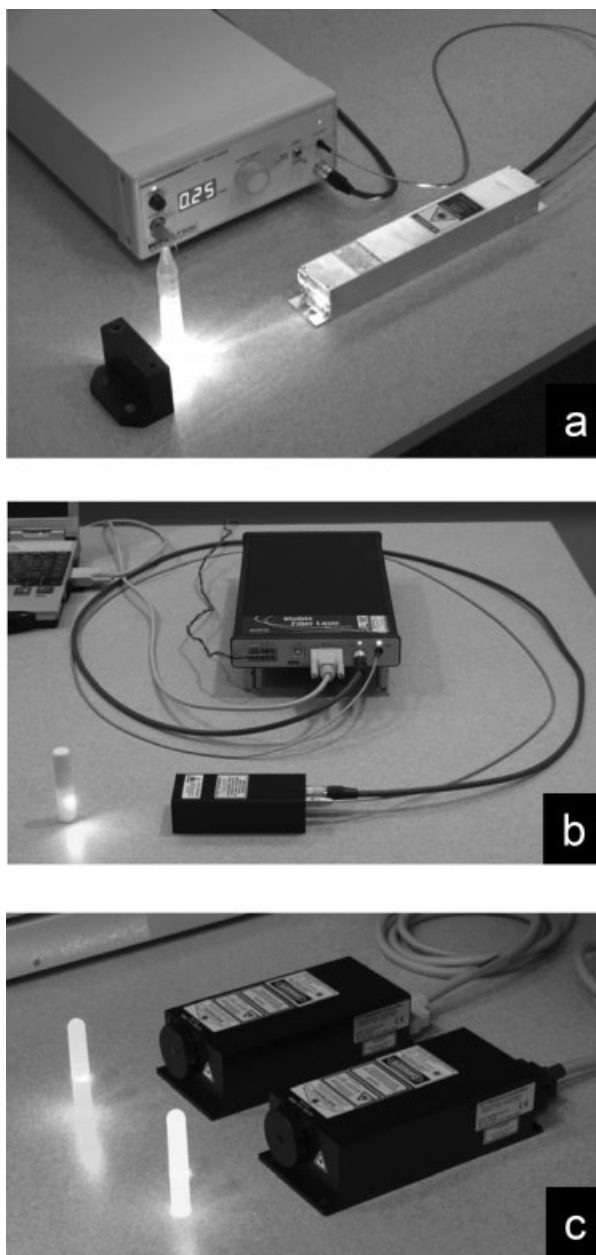


Figure 1. Yellow and orange laser sources. (a), DPSS 580 nm fiber laser, early prototype (MPB Communications); (b), DPSS 592 nm fiber laser, production prototype (MPB Communications); (c), DPSS 558 nm and 589 nm lasers (NEL America).

A variety of yellow, orange, and red excited fluorochromes were then analyzed using this series of lasers. Analysis of Texas Red and Alexa Fluor 594 labeled EL4 cells is shown in Figure 3. The values on the histograms in Figure 3 constitute a sensitivity index or metric calculated from the median background and signal values, accounting for the error, spread and slope of the background peak, and provides a better estimation of instrument sensitivity and laser performance than median intensities alone (13,14). The 589 and 592 nm sources give much better excitation than the 558, 561, or 580 nm

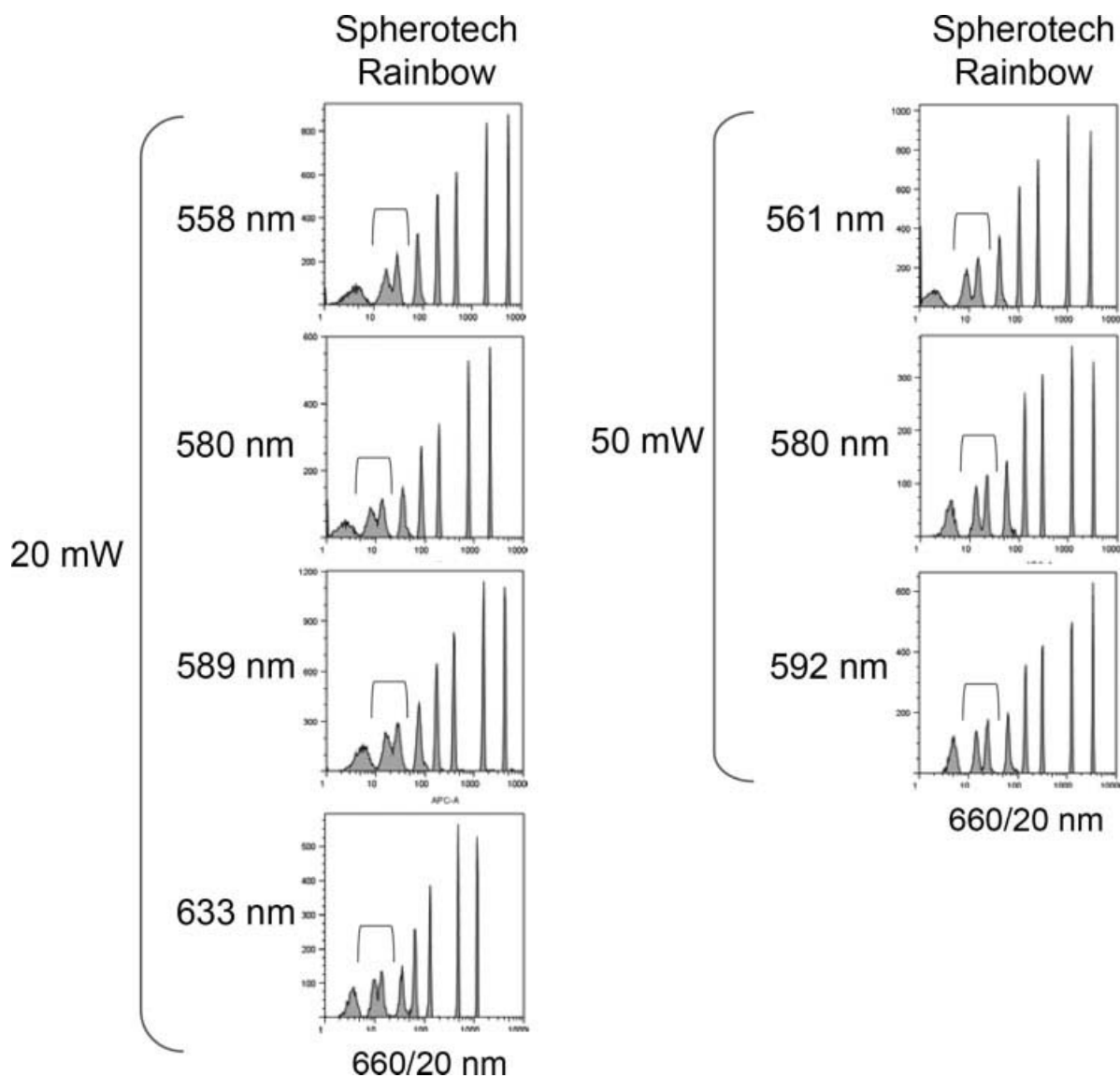


Figure 2. Laser alignment and instrument sensitivity. Spherotech Rainbow eight peak microspheres were analyzed on the BD Biosciences LSR II using the indicated laser sources, emitting at the indicated power level. Red brackets show the resolution between the two dimmest nonblank microsphere populations. Microspheres were detected through a 660/20 nm bandpass filter.

sources based on the sensitivity index. Increasing power level improved fluorochrome detection levels even at nonoptimal excitation wavelengths, with the 580 nm source at 50 mW approaching the sensitivity level of the 592 nm source at 20 mW. However, the 592 nm source at higher power levels, unsurprisingly, gave the best results.

Allophycocyanin (APC) and its tandem conjugates are traditionally excited with red HeNe or diode sources. However, the excitation/emission spectra of APC suggested that shorter orange sources would also provide adequate excitation, with the added utility of providing simultaneous Texas Red and Alexa Fluor 594 excitation. Excitation of APC and its tandems with 580, 589, 592 sources is shown in Figure 4. Interestingly, the 592 nm source at 50 mW excited APC at a

level comparable to that observed for a lower power 633 nm source (2055.3 for the 633 nm versus 2294.4 for the 592 nm), although its ability to excite Alexa Fluor 647 (which has a more red-shifted excitation curve) was still lower. The 592 nm at 50 mW also excited the tandem dye APC-Cy7 well in comparison with the lower power 633 nm (1262.7 for the 592 nm versus 822.2 for the HeNe)

Expressible fluorescent proteins (FPs) represent another class of important fluorescent probes with enormous significance in biomedical research. The development of DsRed, mRFP, and the subsequently derived RFPs with green and yellow excitation maxima has increased the need for flow cytometry lasers with emissions longer than 488 nm. While 532 nm green and 561 nm green–yellow sources are proving

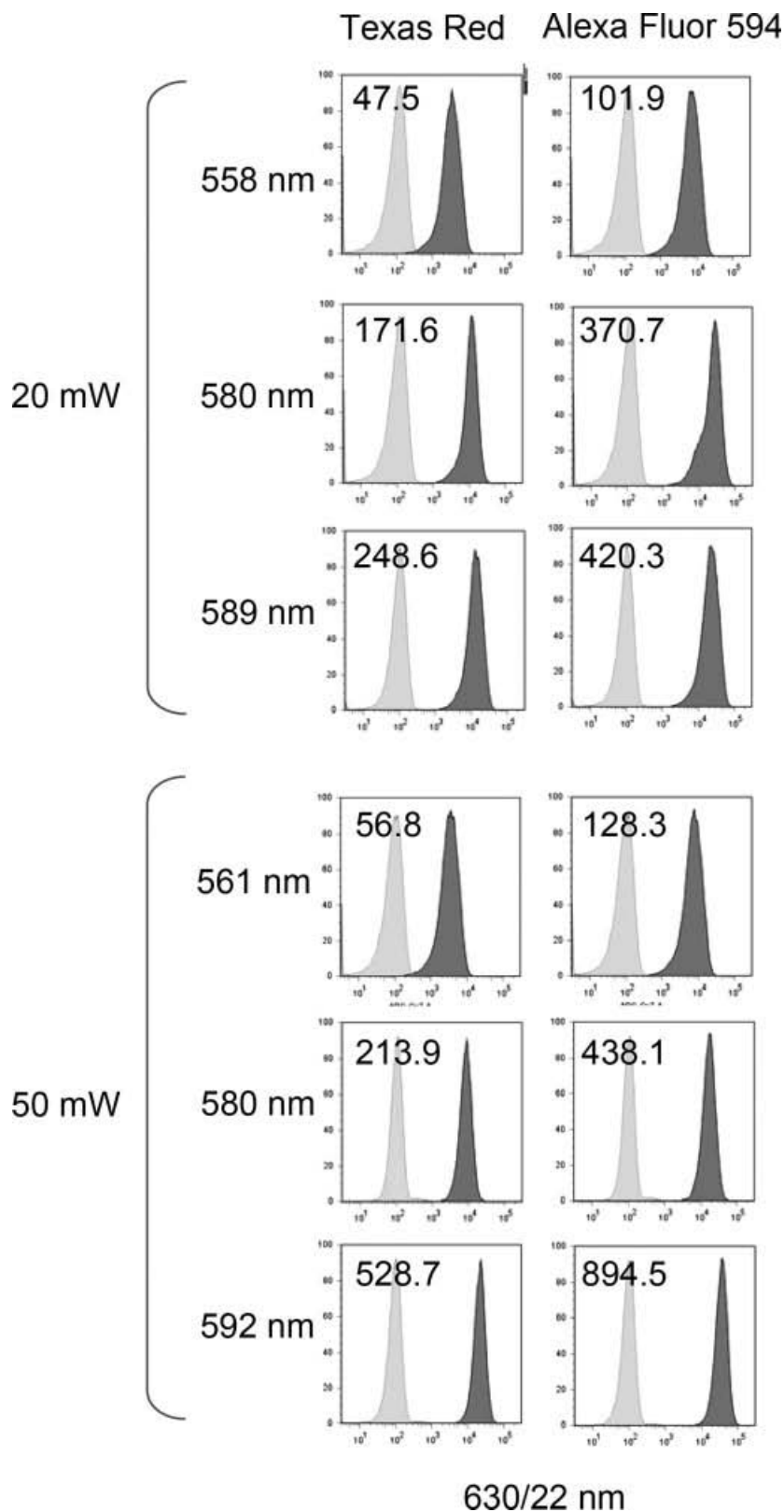


Figure 3. Texas Red and Alexa Fluor 594. EL4 mouse thymoma cells were labeled with biotin-anti-CD44 antibody followed by streptavidin conjugated to either Texas Red or Alexa Fluor 594, and analyzed on the LSR II using the indicated laser and power level. The sensitivity indices (SI-1, see Materials and Methods) are indicated on each histogram. Fluorescence was detected through a 630/22 nm bandpass filter. The 20 mW and 50 mW samples were analyzed in separate experiments. The 20 mW samples were analyzed at PMT voltage between 5 and 15 V greater than the 50 mW samples to normalize background medians.

useful for exciting the shorter wavelength FPs, proteins with longer excitation wavelengths such as mCherry ($\lambda_{EX} = 587$ nm, $\lambda_{EM} = 610$ nm), mPlum ($\lambda_{EX} = 592$ nm, $\lambda_{EM} = 645$ nm), and

the new bright red FP mKate ($\lambda_{EX} = 588$ nm, $\lambda_{EM} = 635$ nm) optimally require longer wavelength laser sources (12,15). This is shown in Figure 5 for HcRed ($\lambda_{EX} = 590$ nm, $\lambda_{EM} = 649$ nm)

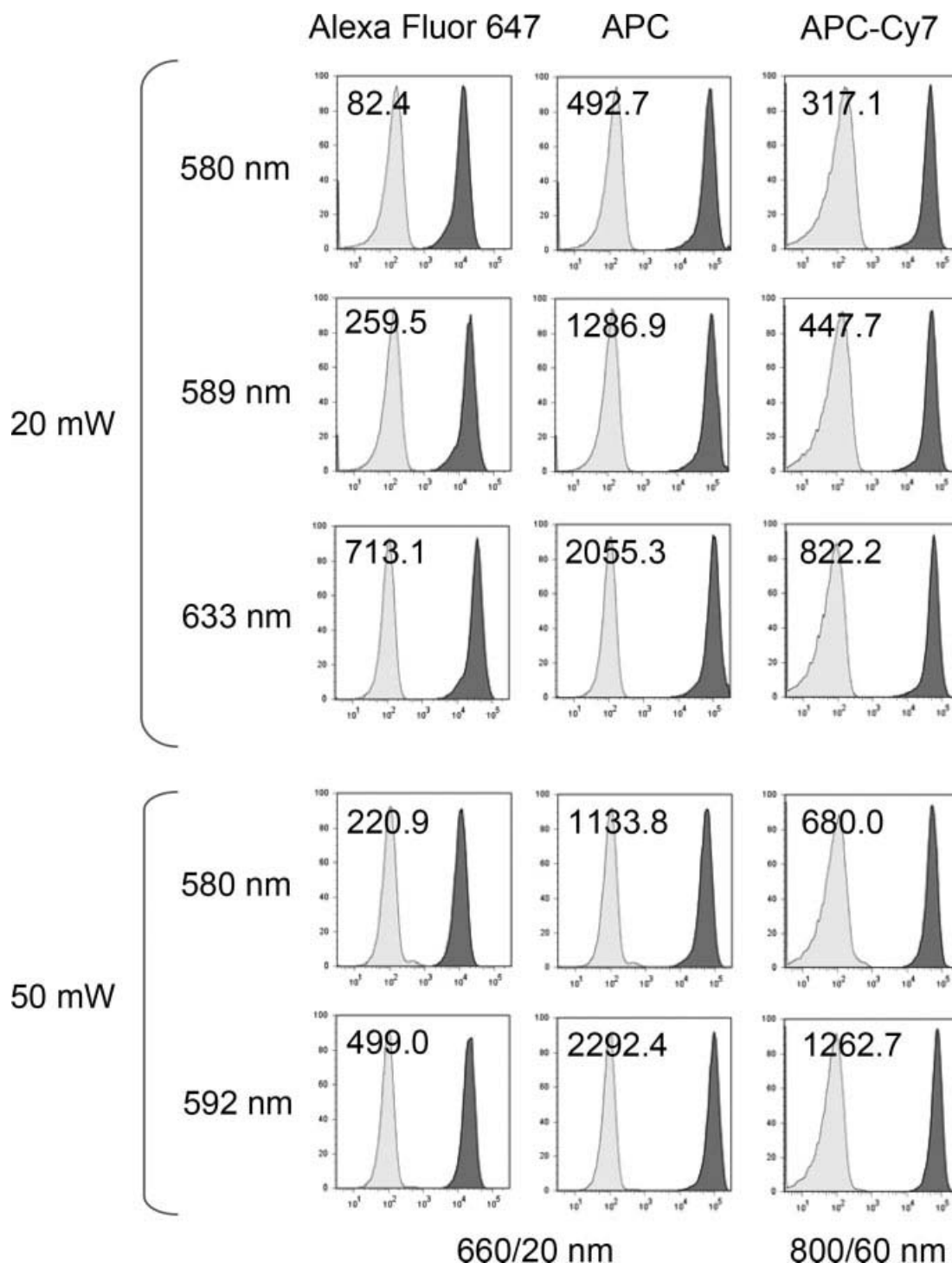


Figure 4. Alexa Fluor 647, APC and APC tandem conjugates. EL4 mouse thymoma cells were labeled with biotin-anti-CD44 antibody followed by streptavidin conjugated to the indicated fluorochromes, and analyzed on the LSR II using the indicated laser and power level. The sensitivity indices (SI-1, see Materials and Methods) are indicated on each histogram. Bandpass filters for each fluorochrome are indicated at the bottom of the histogram panels. The 20 and 50 mW samples were analyzed in separate experiments. The 20 mW samples were analyzed at PMT voltage between 5 and 15 V greater than the 50 mW samples to normalize background medians.

and the newly reported dimerized version of mKate, Katushka ($\lambda_{\text{EX}} = 588 \text{ nm}$, $\lambda_{\text{EM}} = 635 \text{ nm}$) (15), where 559, 580, 592, and 633 nm power-matched sources were used to excite bacteria

expressing the relatively dim protein HcRed (left column), and the far brighter Katushka (right column). Excitation at 561 nm was clearly suboptimal, while 580 and 592 nm at the

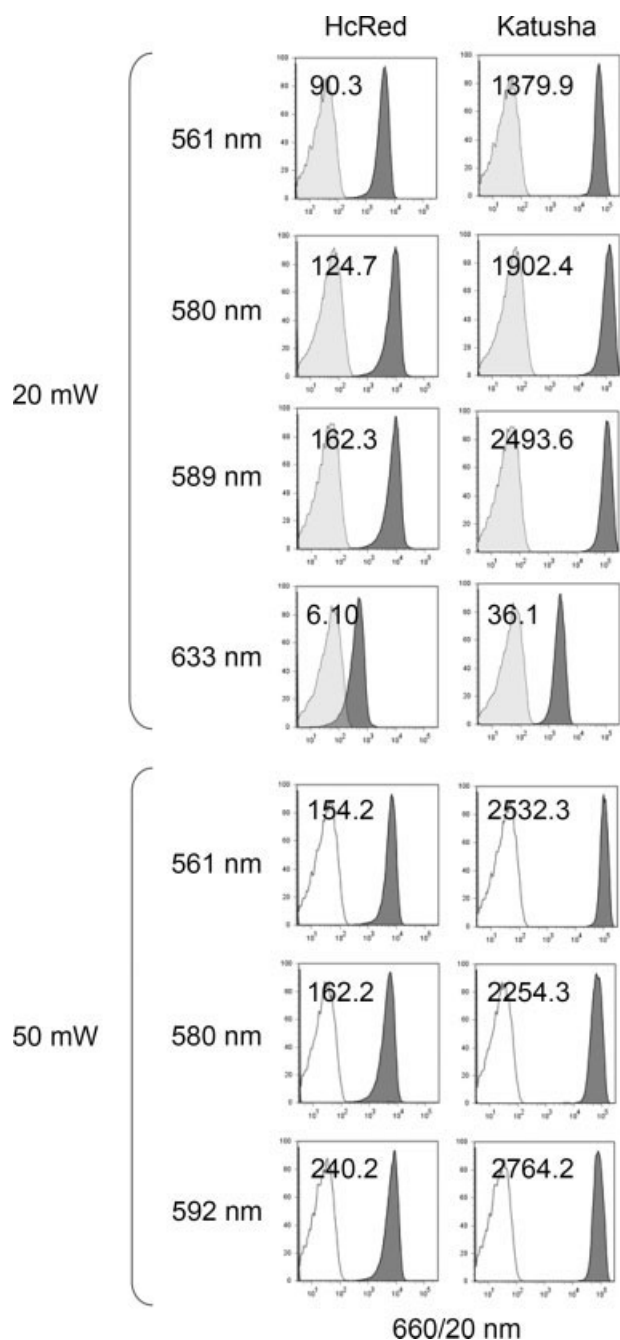


Figure 5. HcRed red fluorescent protein. Bacteria cells expressing empty vectors, HcRed or Katushka were analyzed on the LSR II using the indicated laser and power level. The sensitivity indices (SI-1, see Materials and Methods) are indicated on each histogram. Fluorescence was detected through a 660/20 nm bandpass filter. The 20 mW samples were analyzed at PMT voltage between 5 and 15 V greater than the 50 mW samples to normalize background medians.

same power level improved excitation of both probes considerably.

The 580 and 592 nm fiber lasers were capable of achieving power levels in excess of 200 mW, high for a cuvette instrument but very applicable for stream-in-air cytometers such as

the BD Biosciences FACS Vantage DiVa, Dako MoFlo, or Beckman Coulter Altra. The 592 nm fiber laser were tested on a FACS Vantage DiVa system in Figure 6, at power levels up to 200 mW. Consistent with the previous cuvette cytometer data, the 592 nm source gave excellent excitation of APC, APC-Cy7, and Alexa Fluor 647. Interestingly, both APC and Alexa Fluor 647 saturated at lower power levels; in fact, increasing the power to from 100 to 200 mW slightly decreased the signal-to-background ratio.

DISCUSSION

These results indicate that recently developed yellow and orange laser sources could be successfully integrated into commercial flow cytometers and were useful for exciting a variety of biologically relevant fluorescent probes. Although yellow and orange laser systems have been previously used in flow cytometry, their large size (ion laser pumped dye head systems) or low power levels (yellow and orange HeNe lasers) have severely limited their usefulness. Recent developments in laser technology have produced several DPSS conventional and fiber lasers that emit in the 580–595 nm range, of sufficiently small size yet with power levels to permit both incorporation into small benchtop systems, and also to be used in power-demanding stream-in-air systems. The results in this study demonstrated that yellow and orange lasers were ideal for exciting Texas Red and Alexa Fluor 594, two useful immunophenotyping fluorochromes that see extensive use in epifluorescence and confocal microscopy but little use in flow cytometry due to lack of a good excitation source. The orange 592 nm laser at higher power levels could excite APC and its tandems at levels comparable to traditional red HeNe and diode sources and were adequate for Alexa Fluor 647 detection. As previously demonstrated for mCherry and mPlum, these lasers were particularly useful for the excitation of RFPs such as HcRed, where red HeNe and diode sources are completely inapplicable (12). The ability to incorporate yellow and orange laser sources into flow cytometers is likely to facilitate the screening and discovery of new long-wavelength RFPs by flow.

The development of practical yellow and orange lasers closes an important gap in the range of excitation sources available for biomedical analysis. Until now, the 570–600 nm bandwidth was the only wavelength range not adequately covered by a practical laser source. Until very recently, diode and DPSS laser sources existed from the near-UV (375 nm) up through the violet and blue (400–450 nm), and from the blue–green to green–yellow (475–561 nm). A significant excitation gap existed beyond this point, resuming with the red diode laser at 635 nm. Generation of a yellow or orange laser line required either a gas laser pumped rhodamine 6G dye head, a large, expensive and complex laser system, or a yellow or orange HeNe laser, which operate at extremely low power levels. None of these options are practical for most flow cytometry systems. The development of yellow and orange lasers practical for flow cytometry now permits analysis of virtually any fluorescent probe excited within the visible spectrum. The ability to provide almost any wavelength of visible light for flow cytometry gives us tremendous flexibility in experimental

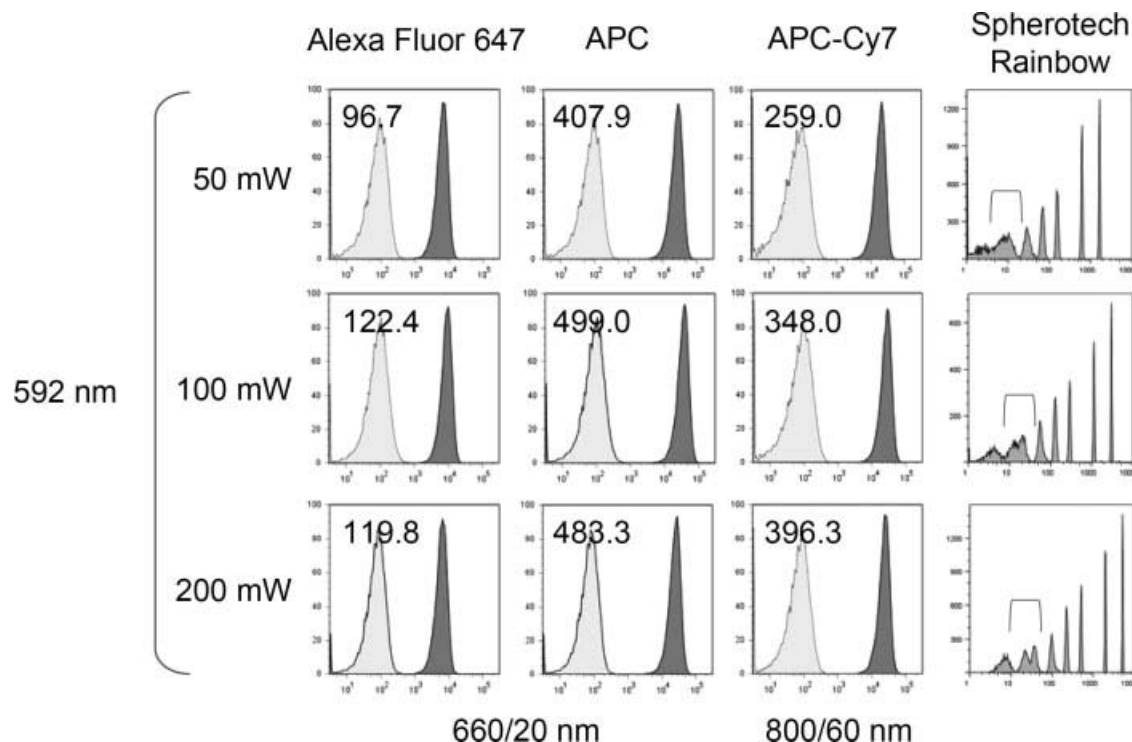


Figure 6. Stream-in-air flow cytometry. EL4 cells labeled with APC, Alexa Fluor 647, or APC-Cy7 were analyzed on a BD FACS Vantage DiVa using the DPSS 592 nm laser emitting at 50, 100, or 200 mW. Spherotech eight-population Rainbow microspheres profiles are also shown (right column). The sensitivity indices (SI-1, see Materials and Methods) are indicated on each histogram; this data is not directly comparable to the preceding LSR II data, however, since a different instrument platform was used. Fluorescence was detected through a 660/20 nm bandpass filter for APC and Alexa Fluor 647 and a 800/60 nm for APC-Cy7. The 50, 100 and 200 mW analyses differed in PMT voltage by 20 V each.

design; we are far less limited by our excitation sources, and can utilize fluorescent probes previously avoided for flow cytometric analysis. Rather than designing our assays based on the limitations of our instrumentation, we can now design our instrumentation to accommodate our assays.

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