DPSS Yellow-Green 561-nm Lasers for Improved Fluorochrome Detection by Flow Cytometry

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Introduction: Blue-green 488-nm laser sources are widespread in flow cytometry but suffer some drawbacks for cell analysis, including their excitation of endogenous proteins (resulting in high cellular autofluorescence) and their less-than-optimal coincidence with the excitation maxima of commonly used fluorochromes, including the phycoerythrins (PE). Longer wavelength lasers such as green helium–neons and, more recently, diode-pumped solid state (DPSS) 532-nm sources have previously been employed to overcome these difficulties and improve overall sensitivity for PE. In this study, we evaluate an even longer wavelength DPSS 561-nm for its ability to improve PE and DsRed fluorescent protein detection sensitivity.

Methods: A DPSS 561-nm laser emitting at 10 mW was mounted onto a BD LSR II. Mouse thymoma cells labeled with cell surface marker antibodies conjugated to the R- and B-forms of PE were analyzed and compared with conventional 488-nm excitation using the same bandpass filters and signal travel distances. A similar analysis was carried out with cell lines expressing the red fluorescent protein DsRed, several green-yellow excited low molecular weight fluorochromes, and a rhodamine-based caspase substrate. Additionally, cells labeled with PE and co-labeled with fluorescein or simultaneously expressing green fluorescent protein (GFP) were analyzed to determine if PE excitation at 561 nm with simultaneous fluorescein/GFP detection was feasible.

Results: The DPSS 561-nm laser gave a several-fold improvement in the fluorochrome to autofluorescence ratios between PE-labeled cells and unlabeled controls. Analysis of cells expressing the fluorescent protein DsRed with the DPSS 561-nm source gave a 6–7-fold improvement in sensitivity over 488-nm excitation, and gave excellent excitation of yellow-green excited fluorochromes and rhodamine-based physiological probes. Yellow-green laser light also caused virtually no impingement on the spatially separated fluorescein/GFP detector, a significant problem with green laser sources, and also allowed simultaneous analysis of GFP and PE with virtually no signal overlap or requirement for color compensation.

Conclusions: DPSS 561-nm laser excitation gave significantly improved sensitivity for both PE-labeled and DsRed expressing cells, with little contamination of a typical fluorescein/GFP detector. Published 2005 Wiley-Liss, Inc.

Key terms: solid state laser; yellow-green laser; phycoerythrin; DsRed

INTRODUCTION

Argon-ion or solid state lasers emitting at 488 nm have traditionally been used as the primary laser sources for flow cytometers. While very useful for multicolor analysis, 488-nm excitation gives suboptimal excitation of the phycoerythrin (PE) fluorochromes, which have excitation maxima in the green to yellow range; the R-form of PE has an excitation maxima of 565 nm, and its absorption at 488 nm is close to 50% maximum (1,2). Blue-green excitation also gives significant excitation of several endogenous molecules, including the flavins, resulting in strong cellular autofluorescence (3). These two factors result in good but somewhat suboptimal sensitivity for PE on conventional flow cytometers, and decreased fluorochrome to autofluorescence ratios because of elevated autofluorescence backgrounds. Efficient excitation of fluorescein at

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and the extremely high quantum efficiency of PE have generally made this compromise acceptable for many immunolabeling applications; however, detection of low-density antigens by PE would certainly benefit from a more efficient excitation system for this widespread fluorochrome.

Longer wavelength lasers, such as DPSS 532 nm, have been proposed as replacements for traditional 488-nm sources in some instruments to reduce these problems. Diode-pumped solid state (DPSS) lasers emitting at 532 nm have provided a small, efficient, cost-effective solution for this application. Several commercial flow cytometers now utilize these sources for the excitation of PE, both in cellular immunolabeling and on microspheres for multiplex analysis of cytokines and other proteins. DPSS 532-nm excitation gives enhanced PE, and PE tandem conjugate excitation with reduced cellular autofluorescence, significantly increasing the sensitivity of PE-based flow cytometry applications (4; Perfetto and Roederer, personal communication). Green lasers are also useful for the excitation of several expressible fluorescent proteins (including DsRed and HcRed) (5–7). Green sources also make accessible for flow cytometry a variety of green-excited low molecular weight fluorochromes that are poorly excited at 488 nm, including rhodamine and tetramethylrhodamine, Cy3 and Alexa Fluor 532, 546, 555, and 568 (8).

Nevertheless, DPSS 532-nm excitation has a significant drawback for multicolor flow cytometry, namely its close proximity to the emission bandwidth of fluorescein and green fluorescent protein (GFP) (9). While most modern multilaser flow cytometers employ spatially separated lasers and signal paths to reduce signal “crosstalk” and laser light contamination between detectors, green laser light on a multilaser instrument cannot be completely isolated from a fluorescein or GFP detector aligned to a 488-nm signal path. The resulting “contamination” of green laser light into the fluorescein or GFP filter, although usually small, can cause a significant amount of background “noise,” making analysis of fluorescein or GFP on a green laser equipped instrument very problematic. Several strategies have been employed to overcome this problem, including better isolation of the laser/signal paths, insertion of green restriction bandpass or notch filters in the fluorescein/GFP signal path to block laser contamination, or the use of shorter fluorescein/GFP bandpass filters to avoid the 532-nm line (Perfetto and Roederer, personal communication). Although all of these approaches have met with some success, they can all result in a loss of sensitivity due to the addition of an additional optical element. An ideal solution to this problem would permit the enhanced sensitivity of a long-wavelength laser source for PE while retaining fluorescein and GFP at their normal sensitivity level.

In this study we have evaluated a longer wavelength DPSS laser emitting at 561 nm for its ability to excite PE and other fluorochromes. The 561-nm laser line is closer to the excitation maxima of both R-PE and DsRed; DPSS 561-nm lasers were found to excite these fluorochromes with considerably better efficiency than 488-nm sources, and were also useful for the excitation of green-yellow excited low molecular weight fluorochromes. In addition, their spectral separation from the fluorescein/GFP range permitted simultaneous analysis of these probes with no additional optical modifications.

**MATERIALS AND METHODS**

**Cells and Standards**

EL4 thymoma cells were obtained from the ATCC (Manassas, VA) and passaged in complete RPMI-1640 media (containing 10% fetal bovine serum (FBS) supplemented with l-glutamine and penicillin/streptomycin). Carmine Linear Check linearity microsphere arrays ( Molecular Probes, Eugene, OR) were used to assess instrument alignment and sensitivity as described previously (4). SP2/0 and NIH-3T3 cell lines expressing DsRed (BD Biosciences Clontech, Palo Alto, CA) were generated as previously described (5–7). Both cell lines were maintained in complete D-MEM media similarly supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were prepared from umbilical cord tissue by digestion with 0.1% collagenase ( Worthington Biochemical, Freehold, NJ) and were propagated through five passages in M199 medium ( Gibco/BRL, Gaithersburg, MD) supplemented with 20% newborn calf serum, 5% human AB serum, 1.6 mM l-glutamine, 25 ng/ml porcine heparin, 50 ng/ml ascobate, and 15 g/ml endothelial cell growth supplement ( Sigma Chemical, St Louis, MO) (10).

**Sample Preparation**

EL4 cells were washed once with PBS containing 2% FBS (no sodium azide) and incubated with biotin-conjugated antibody against either mouse CD44 or CD90 (CalTag Laboratories, San Diego, CA). Cells were then washed with PBS/FBS and labeled with streptavidin conjugates of the R- or B-forms of PE (Prozyme), fluorescein, lissamine rhodamine, or Alexa Fluor 568 ( Molecular Probes) followed by paraformaldehyde fixation and analysis within 24 h. DsRed expressing SP2/0 and NIH 3T3 cells were washed with PBS and fixed with paraformaldehyde fixation followed by analysis within 24 h. For apoptosis measurement, using fluorogenic caspase assays, EL4 cells were incubated with cycloheximide for 4 h, washed and labeled with the rhodamine-based caspase 3 substrate PhiPhiLux G2D2 (Oncoimmunin, Gaithersburg, MD) for 45 min at 37 °C, followed by counterstaining with Hoechst 33258 at 1 g/ml and analysis within 1 h (10–12). HUVECs were infected with a GFP expressing lentivirus ( Casellas and Baltimore, unpublished data). After 48-h infection, cells were detached with 2 mM EDTA, washed and labeled with a PE-conjugated antibody against NRP-1 ( Miltenyi Biotec, Auburn CA), fixed as above, and analyzed (13).

**Flow Cytometry**

Cells were analyzed on a BD Bioscience LSR II (San Jose, CA) equipped with a DPSS 488-nm laser emitting at 20 mW (Coherent) and a DPSS 561-nm laser emitting at...
10 mW (Melles Griot, Carlsbad, CA). The DPSS 561-nm laser occupied the same position normally occupied by the red helium–neon laser supplied with the default instrument configuration, focused on the fourth signal pinhole position (with the 488-nm laser occupying the first position). The DPSS 561-nm laser was attached to a retrofitted mounting platform on the LSR II with two-axis translational capability, and the beam steered with a 100% mirror mounted on a three-axis gimbaled filter mount (Fig. 1a). For some experiments, the DPSS 561-nm laser was replaced with a DPSS 532-nm laser emitting at 40 mW (Power Technology, Alexander AR and Laser-Compact, Moscow, Russia). Laser beam profiles were measured using a WinCamD profiling system (DataRay, Boulder Creek, CA) utilizing a 6.3 x 4.7 mm² Sony CCD chip with a neutral density filter blocker (laser profile shown in Fig. 1b). Laser alignment was carried out using Carmine Linear Check microspheres with relative fluorescent intensities of 10%, 2%, 0.4%, 0.1%, and 0.02% of an arbitrary 100% maximum standard as previously described (4). Contamination of the fluorescein detector on the LSR II by 532- or 561-nm laser light was measured using fluorescein low-range MESF microspheres (Bangs Laboratories) analyzed through a standard 530/30-nm filter. Carmine microsphere, R-PE, B-PE, DsRed, lissamine rhodamine, Alexa Fluor 568, and PhiPhiLux G2D2 labeled samples were analyzed with a 610/20-nm bandpass filter, using the same filter sequentially for DPSS 488- and 561-nm measurements. The 610/20-nm bandpass filter was used to avoid laser light spillover from the 561-nm laser source. All optical filters were obtained from Omega (Brattleboro, VT) and mounting components from Newport Corp. (Irvine, CA). Data were acquired using the digital DiVa acquisition software package (BD Biosciences) analyzed with WinMDI version 2.8 (Joseph Trotter, BD Biosciences).

RESULTS
Alignment and Sensitivity
In this study, a DPSS 561-nm laser was installed on a BD LSR II flow cytometer, and assessed for its utility as a flow cytometric excitation source. Figure 1a shows the installation scheme, with the laser mounted on a two-axis translational stage, and the beam reflected at a 90° angle with a 100% reflectance laser mirror positioned in a gimbaled filter mount with three axes of rotational freedom. The laser beam was steered into the laser path normally reserved for a red HeNe laser in the default LSR II configuration, and steered to the flow cell using a 500-nm shortpass dichroic (which simultaneously transmitted the 488-nm laser beam). The 561-nm beam gave a nearly Gaussian distribution as measured by a CCD chip (Fig. 1b). The laser was then aligned using Carmine Linear Check microspheres with six distinct fluorescence intensities. A typical alignment and sensitivity check is shown in Figure 2, where the microspheres were first analyzed on a log scale (Fig. 2a), then in linear scaling with each descending population set at a fixed channel value and the CV measured (Figs. 2b–2e). Laser alignment and instrument sensitivity was routinely assessed by both the relative fluorescent intensities of the individual microsphere populations and by their CVs. Low CVs for the dimmest populations (3.84% for the 0.03% population in this case) were indicative of both good alignment and sensitivity.
R- and B-Phycocerythrins

Green lasers (including DPSS 532-nm and HeNe 543.5-nm sources) have been employed in the past for PE excitation, both as a more optimal excitation source for this fluorochrome and to reduce cellular autofluorescence backgrounds (3,4). Since a 561-nm source corresponds even more closely to the excitation maxima for PE and similarly avoids the excitation spectra for endogenous molecules, such as flavins, a significant improvement in PE detection fluorochrome to autofluorescence ratio would be expected (2,3). This was demonstrated in Figure 3 for the R-form of PE. EL4 mouse thymoma cells were labeled with either biotin-conjugated CD44 (Figs. 3a and 3b) or CD90 (Figs. 3c–3f), followed by R-PE conjugated streptavidin. The labeled samples were then analyzed with both DPSS 488-nm (top row) and 561-nm (bottom row) excitation using the same signal path lengths and bandpass filter. On the basis of relative mean fluorescent intensities (MFIs), the fluorochrome to autofluorescence ratio for specific PE labeling versus background autofluorescence increased from 2- to 4-fold with 561-nm excitation compared to that with 488-nm excitation. In the case of CD90 labeling, gain settings that allowed on-scale visualization of CD90 expression using the 488-nm laser drove the signal off-scale with 561 nm (Figs. 3c and 3d); decreasing the gain equally for both detectors was required to bring the 561-nm excited CD90 signal on-scale (Figs. 3e and 3f).

The B-form of PE possesses somewhat longer excitation peak of 545 nm and is more poorly excited at 488 nm than the R-form (2). However, the recent trend of DPSS green laser installations on small benchtop cytometers has increased the usage of this phycobiliprotein. When compared with R-PE, B-PE was extremely well-excited by the DPSS 561-nm laser (with a greater increase in fluorochrome to autofluorescence) compared with the 488-nm laser (Fig. 4).

DsRed

Similarly, the expressible fluorescent protein DsRed is frequently excited with 488-nm laser sources but is more optimally excited with green or yellow lasers; previous studies have used the green 530 nm and particularly the green-yellow 568-nm lines from krypton-ion lasers for DsRed excitation with good results (5–7). In this study, NIH3T3 and SP2/0 cells constitutively expressing DsRed were analyzed with both 488- and 561-nm lasers on the BD LSR II as above. The DPSS 561-nm laser was an excellent excitation source for DsRed, eliciting fluorochrome to autofluorescence ratios several times greater than those observed with a 488-nm laser on the same instrument (Fig. 5).

Low Molecular Weight Fluorochromes

A number of low molecular weight fluorochromes are available that excite in the green to yellow range, but have not seen widespread usage in flow cytometry because of their long excitation wavelength. Rhodamine and its derivatives (including tetramethylrhodamine), Cy3, and Alexa Fluor 532, 546, and 555 are well-excited by green sources, while yellow-green sources (such as the 568 nm from krypton-ion lasers) can excite lissamine rhodamine and Alexa Fluor 568. The DPSS 561-nm laser was found to excite two of these fluorochromes with longer excitation wavelengths, lissamine rhodamine and Alexa Fluor 568, at least as well as more powerful DPSS 532-nm sources, based on immunolabeling of EL4 cells (Fig. 6).

These low molecular weight fluorochromes see extensive use in microscopy and are often incorporated into physiological probes because of their small size. For example, fluorogenic enzyme substrates frequently employ rhodamine and its derivatives as the incorporated fluorochrome (14); the dearth of green lasers on cytometers has until recently limited the use of these reagents primarily to epifluorescence and confocal microscopy or spectrofluorimetry. In Figure 7, a rhodamine-based substrate for
the apoptosis-associated enzyme caspase 3 (PhiPhiLux G2D2, Oncoimmunin) was loaded into EL4 cells previously treated with cycloheximide for 4 h. The PhiPhiLux G2D2 substrate rhodamine component is quenched in the intact, uncleaved form; upon cleavage by caspase 3, quenching is relieved and rhodamine fluorescence can be detected (10–12). As illustrated in Figure 7, early apoptotic cells with active caspase 3 but low incorporation of the cell-impermeant DNA probes Hoechst 33258 cannot be well-distinguished from viable cells using 488-nm excitation (Figs. 7a and 7b), but are clearly identified by using 561-nm excitation (Figs. 7c and 7d).

Avoidance of Fluorescein Emission Range with 561-nm Excitation

Green DPSS lasers are now being used extensively to excite PE with excellent results (Peretto and Roederer, personal communication). Nevertheless, a key drawback in green DPSS for flow cytometry is their coincidence with the emission bandwidths of fluorescein and GFP. Although variable from instrument to instrument, the optical paths for emission signals cannot be completely isolated from laser light spillover, even when the laser beam and the signal path are spatially separated. As a result, a small amount of green laser light contaminates the signal paths of the 488-nm laser, “spilling over” into the fluorescein/GFP detector and causing unacceptably high levels of background. Optical strategies must therefore be employed to reduce or eliminate laser light spillover into the fluorescein detector, including the use of shorter wavelength bandpass filters, or notch filters or dichroics to block contaminating laser light.

Yellow-green laser excitation, however, does not impinge on the fluorescein detector using a standard 530/30-nm bandpass filter. This is illustrated in Figure 8, where low fluorescence MESF microsphere arrays were analyzed on
the cytometer equipped with either DPSS 532- or 561-nm sources (top and bottom rows, respectively), with the secondary laser either off or on (left and right columns). With a standard 530/30-nm bandpass filter, the presence of 532-nm laser light did not increase the fluorescence of the brightest bead population excessively, but completely destroyed resolution of the dimmer populations; 561-nm laser light had no effect on this resolution. DPSS 561-nm laser excitation is therefore compatible with simultaneous fluorescein or GFP detection, requiring no further instrument optical modifications.

This compatibility had some interesting ramifications for simultaneous fluorescein/GFP and PE analysis, beyond the ability to improve PE sensitivity while still analyzing green-emitting probes. Fluorescein, for example, has considerable emission overlap with PE, requiring electronic compensation to subtract the fluorescein signal from PE (15). The problem is illustrated in Figures 9a–9c, where a mixture of EL4 cells labeled with fluorescein and PE conjugated antibodies against CD90 were analyzed with a 488-nm laser to excite both fluorochromes. The fluorescein emission overlaps significantly with PE

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as expected (Fig. 9a), requiring a considerable level of postanalysis color compensation to subtract fluorescein “bleedover” from the PE detector (Fig. 9b). When fluorescein is excited at 488 nm and PE at 561 nm with spatially separated beams, however, no compensation of fluorescein from the PE detector is required. The 561-nm laser

**Fig. 7.** DPSS 561-nm excitation of PhiPhiLux G2D2 caspase 3 substrate. EL4 cells were left untreated (a and c) or treated with cycloheximide at 50 μg/ml for 4 h (b and d), loaded with the fluorogenic caspase substrate PhiPhiLux G2D2, counterstained with Hoechst 33258 and analyzed on the BD LSR II using either 488-nm (a and b) or 561-nm (c and d) excitation. All two-parameter data are displayed as ungated for scatter, with PhiPhiLux G2D2 on the X-axis versus Hoechst 33258 exclusion on the Y-axis. Corresponding one-parameter histograms for PhiPhiLux G2D2 fluorescence are gated for the region displayed on the corresponding two-parameter plot, with percentage apoptotic cells shown.

**Fig. 8.** Exclusion of DPSS 561-nm laser light from fluorescein/GFP detector. MESF fluorescein low range microspheres (Bangs Laboratories) were analyzed on the BD LSR II in the default fluorescein/GFP detector (with 530/30-nm bandpass filter) with installation of either DPSS 532-nm (top row) or 561-nm (bottom row) lasers, with each laser either OFF (left column) or ON (right column).
does not excite fluorescein, and this laser wavelength does not impinge on the fluorescein detector as described earlier.

Eliminating the requirement for color compensation is even more useful for red-shifted mutants of GFP (such as EGFP), which can overlap the PE emission spectrum to a greater extent than can fluorescein (4,5,9). In Figures 9d-9g, HUVECs expressing GFP were analyzed on the BD LSR II with (d) 488-nm excitation only, no compensation; (e) 488-nm excitation with PE detector = %GFP compensation = 10%; (f) spatially separated 488-nm excitation of GFP and 561-nm excitation in the PE channel with no compensation. (g) HUVECs transduced with GFP and immunolabeled with PE antibody against NP-1, analyzed with spatially separated 488-nm excitation of GFP and 561-nm excitation of PE with no compensation; and (h) GFP only control for (g).

FIG. 9. Fluorescein or GFP overlap into PE signal with spatially separated DPSS 561-nm excitation. (a–c) EL4 cells were labeled with biotin–anti-CD90 followed by either FITC- or R-PE-streptavidin. Mixtures of unlabeled, fluorescein- and R-PE-labeled cells were then analyzed on the BD LSR II with (a) 488-nm excitation for both probes, no compensation; (b) 488-nm excitation of both probes, PE-%fluorescein compensation = 20%; or (c) spatially separated 488-nm excitation of fluorescein and 561-nm excitation of R-PE with no compensation. Fluorescein-%PE compensation was 0.5% for all samples. (d–h) lentivirus-transduced HUVECs expressing GFP were analyzed on the BD LSR II with (d) 488-nm excitation only, no compensation; (e) 488-nm excitation with PE detector = %GFP compensation = 10%; (f) spatially separated 488-nm excitation of GFP and 561-nm excitation in the PE channel with no compensation. (g) HUVECs transduced with GFP and immunolabeled with PE antibody against NP-1, analyzed with spatially separated 488-nm excitation of GFP and 561-nm excitation of PE with no compensation; and (h) GFP only control for (g).

DISCUSSION

In this study, yellow-green laser excitation of PEs (both R- and B-forms) and DsRed was found to give superior fluorochrome to autofluorescence ratios over standard 488-nm excitation, a situation predicted by their excitation spectra (2). The degree of improvement was similar to that previously observed for green laser sources such as HeNe and DPSS, resulting from the combined effects of improved excitation and reduced cellular autofluorescence using these longer wavelength sources. Yellow-green excitation gave an interesting advantage over green sources in its spectral distance from the excitation range of fluorescein and GFP, causing essentially no laser spillover into the spectral range of standard bandpass filter for these probes. This allowed simultaneous analysis of fluorescein or GFP and PE using standard green bandpass filters, with no need for notch blocking elements or other modifications to prevent contamination of the green
detector by laser light. Since excitation at 561 nm gives virtually no fluorescein or GFP excitation, and does not impinge on the green detector, fluorescein or GFP and PE could be analyzed simultaneously with no compensation required for subtraction of the green from PE signal.

Spectrally separated excitation of GFP (or fluorescein) and PE therefore gave (1) better sensitivity for PE, and, at the same time (2) the ability to simultaneously measure fluorescein or GFP with no additional optical modifications, and most interestingly (3) virtually no fluorescence overlap between the two probes, eliminating the need for compensation. Although color compensation is used widely and effectively to subtract spectral overlap of fluorescent probes, the subjective nature of manual compensation settings (often resulting in “overcompensation” or “undercompensation” of multicolor data) can cause difficulties in data analysis, particularly when dealing with weakly expressed antigens (15). Wherever possible, reduction or elimination of compensation is always desirable in multicolor analysis to reduce the possibility of compensation becoming an artifact in data analysis.

This study also illustrates the utility of small solid-state laser sources in flow cytometry. Diode and DPSS laser sources are becoming common fixtures in flow cytometers (4,16,17); as the available wavelengths increase, they should find many applications both in specialized fluorescence applications and in more routine applications (such as the ones illustrated in this study) as improved excitation sources for.

LITERATURE CITED