Lasers for Flow Cytometry

Howard M. Shapiro[1] and William G. Telford[2]

[1]The Center for Microbial Cytometry, West Newton, Massachusetts
[2]Experimental Transplantation and Immunology Branch, National Cancer Institute, Bethesda, Maryland

ABSTRACT

Lasers are the principal light sources for flow cytometers. Most modern benchtop instruments feature air-cooled lasers emitting 10 to 25 mW of light at 488 nm. This unit covers the various types of lasers available and the qualities that make them suitable or unsuitable for use in flow cytometers. Also included is a discussion of future directions, particularly in the development of solid-state devices. *Curr. Protoc. Cytom.* 49:1.9.1-1.9.17. © 2009 by John Wiley & Sons, Inc.

Keywords: flow cytometry • Gaussian beams • illumination optics • lasers

INTRODUCTION

Most fluorescence flow cytometers use one or more lasers as light sources; in fact, the Bio/Physics Systems Cytofluorograf flow cytometer, introduced in 1970, was the first commercial product to incorporate an argon-ion laser. Although many benchtop instruments still in service feature similar air-cooled argon-ion lasers, emitting 10 to 25 mW of light at 488 nm, newer flow cytometers typically use a variety of solid-state lasers as light sources.

The process by which a laser beam, typically 0.5 to 2.0 mm in diameter, is focused to a smaller elliptical spot to illuminate the sample stream is discussed in *UNIT 1.6*; this unit covers lasers themselves and the qualities that make them suitable or unsuitable for use in flow cytometry. Almost all of the lasers discussed in this unit can also be used for other laser-based imaging technologies such as scanning cytometry (*UNIT 2.10*) and confocal microscopy (*UNIT 2.8*).

WHY LASERS OR ARC LAMPS ARE NEEDED AS LIGHT SOURCES FOR FLOW CYTOMETRY

Flow cytometric measurements of scattered light and fluorescence emission are made during the few microseconds in which a cell passes through the illuminating beam. Basic physics dictates that the total number of photons scattered and emitted by a particle cannot exceed the number of photons incident upon it, and as even the most efficient collection optics capture light over a relatively small solid angle, only a fraction of this total can possibly contribute to a measured signal. As much as a third of the light collected in some spectral regions may be lost in the various dichroic mirrors and filters used to divert light to the detectors. The detectors themselves convert no more than half (diodes) and typically less than one quarter (photomultiplier tubes) of the photons incident on their photocathodes to photoelectrons, which are the source of the signal currents further processed by flow cytometers' electronics.

It is thus necessary that the light source used in a flow cytometer be able to emit a relatively large number of photons per unit time, and further, that it be possible to direct or focus a substantial fraction of those photons into a small volume of the sample stream. The critical characteristics of a light source can be defined quantitatively in terms of its **radiance**, which is the power emitted from, transmitted through, or reflected by a surface, per unit of its area, per unit solid angle; the units of radiance are $W/m^2/sr$ (watts per square meter per steradian).

Extended light sources, a category that includes filament and arc lamps and light-emitting diodes (LEDs) as well as the sun, can emit substantial amounts of power, but their emission is typically dispersed in all directions, i.e., over a solid angle of 4π sr, which represents the surface of a sphere. It is not physically possible to direct more light from an extended source through a small area of space than is emitted from an equivalent area of the source. Thus, while a substantial fraction of the emission from such a source can be collected

Current Protocols in Cytometry 1.9.1-1.9.17, July 2009
Published online July 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471142956.cy0109s49

using parabolic or elliptical reflectors, only a small part of the collected light can be delivered to a cell or a particle of similar size. Flow cytometers that do employ extended sources use high numerical aperture lenses to collect light from the source and to illuminate and collect light from cells in the sample stream. Arc lamps, which have relatively high radiance, are much better suited to flow cytometry than are LEDs or filament lamps, which have substantially lower radiance. The seeming paradox that lower power (e.g., 100-W mercury) arc lamps are better sources for cytometers than lamps of substantially higher power finds an explanation in the smaller lamps' higher radiance, which results from smaller dimensions of the arc itself.

The output of a laser is typically contained in a beam of relatively small diameter (0.5 to 2 mm) with a small angle of divergence; the radiance of a laser is, therefore, extremely high. The submilliwatt lasers used in a supermarket bar code scanner may emit more photons per square meter per steradian than does the surface of the sun. Moreover, all of the power in a laser beam can be focused to a spot with dimensions as small as a few micrometers using a simple lens; for reasons discussed at length in *UNIT 1.6*, the optical systems of flow cytometers usually incorporate crossed cylindrical lenses of different focal lengths to produce an elliptical spot.

HOW LASERS WORK

Stimulated Emission

The word "laser" is an acronym for "light amplification by stimulated emission of radiation." The physical process behind all lasers is **stimulated** or **induced emission**, a type of fluorescence described by Einstein in the early 1900s. In order for any kind of fluorescence to occur, the absorption of a photon must first excite the absorbing atom, ion, or molecule (hereafter, "molecule" will refer to "atom, ion, or molecule"), producing a transition from a lower to a higher electronic energy level. After a brief period of time, usually a few nanoseconds, the molecule typically returns to a lower energy state by emitting a photon with energy less than or equal to that of the absorbed photon. Under most circumstances, only a small minority of the molecules in the illuminated material is in excited states, and the photons emitted from different excited molecules are different in wavelength, phase, and polarization; what occurs is called **spontaneous emission**. As Einstein showed, however, once a molecule has been excited by absorption, the mere presence of a photon or photons of a particular energy in its vicinity increases the probability that it will emit a photon of the same energy (frequency or wavelength), phase, and polarization. Thus, photons can induce or stimulate the emission of like photons.

Stimulated emission becomes more likely as the fraction of the molecules in excited states increases, and can become self-sustaining when there is a **population inversion**, i.e., when the excited molecules outnumber those in the lower energy state. In general, it is difficult to create population inversions for energy transitions between the lowest excited state and the ground state of a molecule, because the ground state is more favorable on thermodynamic grounds according to the Boltzmann law. Most practical lasers emit at a wavelength corresponding to the energy of a transition between a **metastable** higher energy excited state, i.e., one with a relatively long lifetime, and a lower energy excited state. The lasing medium is excited, or **pumped**, by electrical energy or by a high-intensity light source, causing the molecules in the medium to undergo transitions to excited states with energies equal to or higher than that of the metastable state; those at higher energies subsequently drop to the metastable state nonradiatively. Initially, spontaneous emission occurs at a particular laser wavelength as molecules drop from the metastable state to the lower excited state; thereafter, spontaneously emitted photons stimulate the emission of additional photons at the laser wavelength and the process continues. The population inversion required to sustain stimulated emission is maintained because molecules rapidly leave the lower energy state of the laser transition by thermodynamically favorable transitions to excited states of still lower energy or to the ground state. A diagram of the energy levels typically involved in laser action appears as Figure 1.9.1.

Gain, Resonators, and Optical Feedback

While the "l" and the "a" in "laser" stand for "light amplification," an operating laser is more like an amplifier that has been driven into oscillation by application of positive feedback. Most of us are familiar with an audio analogue of this process, in which sound from a microphone placed in front of the speaker drives the audio amplifier into oscillation; the frequency

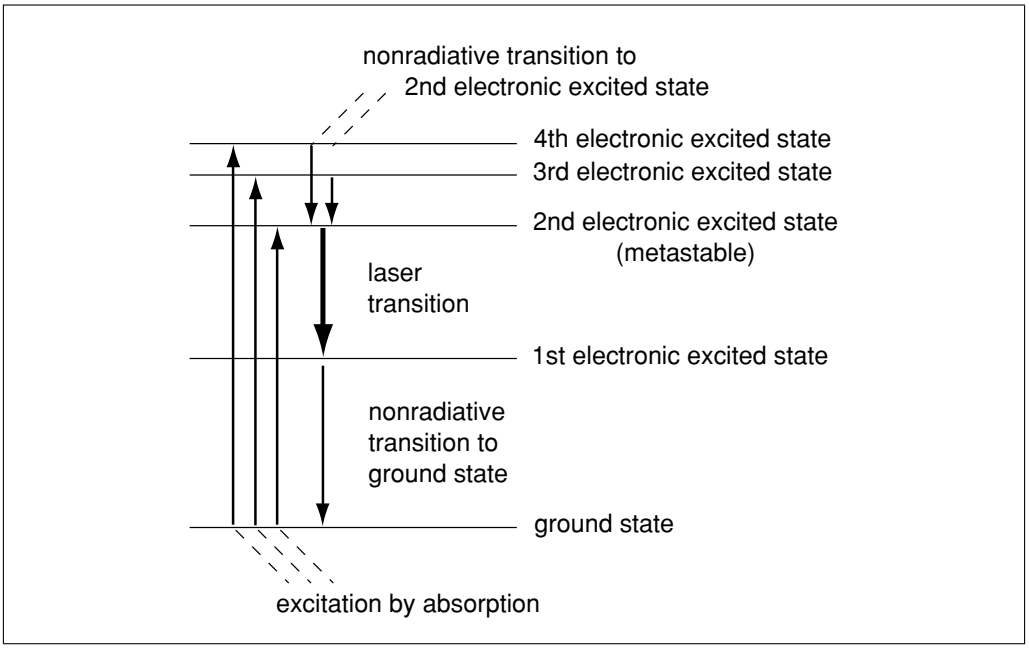

Figure 1.9.1 Transitions between electronic energy states in a typical laser.

of oscillation is a function of the distance between microphone and speaker.

The initiation of stimulated emission in a volume of a suitable material will not in and of itself produce the concentrated, low divergence light beams that characterize lasers and on which so much of their utility depends. It will, instead, result in light emission in all directions, i.e., over a solid angle of 4π sr. This is so because, while the photons produced by stimulated emission travel in more or less the same direction as the stimulating photons, the spontaneously emitted photons responsible for the first round of stimulation do not have any directional preference. It is necessary, therefore, to perform some geometrical and optical manipulations in order to make a usable laser.

First, the volume of lasing medium in which stimulated emission occurs is shaped to produce some directionality of emission. As just mentioned, spontaneously emitted photons are equally likely to be emitted in any given direction, and photons produced by stimulated emission, which follow the paths of the stimulating photons, will therefore also be equally likely to be emitted in any given direction. The probability that one photon will stimulate emission of others in the medium is proportional to the length of the path of the photon in the medium. If the medium were formed into a spherical shape, this average path length would be the same in all directions. The **gain** of the lasing medium, i.e., the number of stimulated photons emitted per unit distance per incident photon, depends predominantly on the quantum mechanical properties, i.e., energy levels and transition probabilities, of the medium. If the gain is high enough, and the intensity of excitation of the medium is sufficient, stimulated emission may be sustained in a spherical volume, but emission will be neither directional nor coherent. These conditions are, incidentally, believed to exist for carbon dioxide molecules in the atmosphere of Mars.

If the lasing medium is shaped into a long, thin cylinder or rod, photons emitted parallel to or at small angles to the axis are more likely to stimulate emission than are photons emitted along or near the radius, because the path of the axial photons is substantially longer. Thus, the geometry of the medium will favor emission along the axis. Making the medium longer will, in general, increase the amount of power that can be obtained.

In many practical lasers, the directional property achieved by shaping the lasing medium is augmented by placing the medium inside a relatively rigid structure or **resonator**, with precisely aligned and spaced mirrors, highly reflective at the desired output wavelengths, mounted at opposite ends. Light emitted along the axis of the resonator is reflected back along the same path again and again, while light at increasingly larger angles to the axis is less and less efficiently reflected back through the medium. Since light produced by stimulated emission is identical in wavelength, phase, and direction to the stimulating light, most of the emission confined within the laser cavity, i.e., the space between the mirrors, will

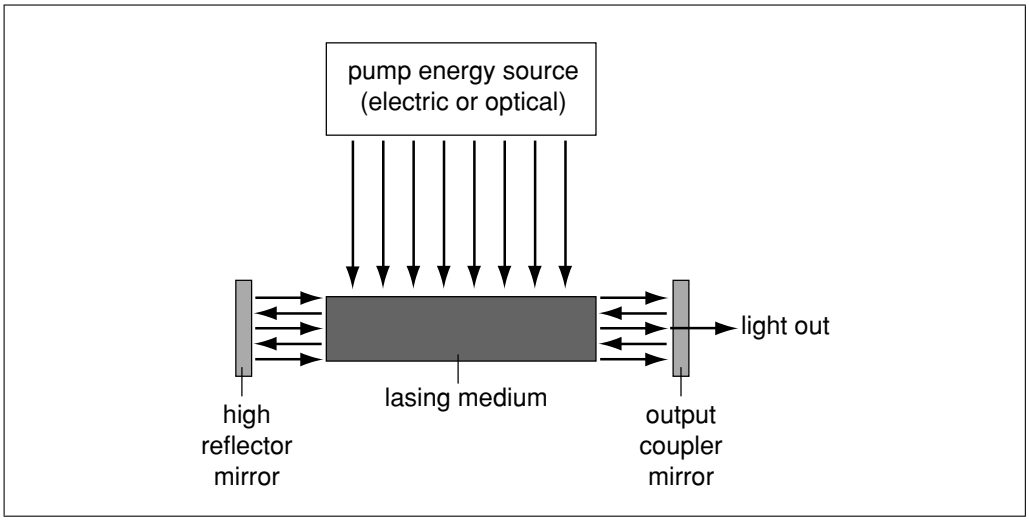

Figure 1.9.2 Schematic diagram of a laser. Brewster windows used to produce polarized output would be placed between the medium and the mirrors; a Littrow prism or wedge used for wavelength selection is typically placed between the medium and the high reflector mirror.

be concentrated along or very near its axis. Laser output is produced by making one of the mirrors able to transmit a small fraction of incident light; the amount of transmission permissible varies with the gain of the medium, which must be high enough to make up for the light lost by transmission outside the cavity and the light lost by absorption within the cavity. A schematic sketch of a laser is shown in Figure 1.9.2.

The spacing between the mirrors is critical. If they are an even number of wavelengths apart, constructive interference will occur between the rays incident on and those reflected from the mirrors, maximizing output. If not, there will be destructive interference, which may be enough to prevent laser action entirely. In medium- and high-power ion lasers, wavelength selection within the spectral range attainable with a single set of mirrors is generally done by insertion of a **Littrow prism** in the cavity between the mirrors. The dispersion of the prism results in light of different wavelengths being refracted at different angles on passage through the prism. At any given position of the prism, only a relatively narrow range of possible emission wavelengths will be reflected along the axis of the laser cavity between the high reflector and output coupler mirrors. Gain in this selected wavelength range will be sufficient to maintain laser action; gain at wavelengths above and below the selected range will not. The emission wavelength is changed by changing the orientation of the prism; this usually involves a vertical angular adjustment. A dispersive element other than a prism, e.g., a grating, etalon, or optical filter, can also be used for wavelength selection.

The resonator can be thought of as analogous to an organ pipe; the length of the pipe, and the effective distance between the mirrors of the resonator, determine the frequency of the standing wave sustained by the structure. In the case of the resonator, this characterizes what is described as the **longitudinal mode** of the laser.

The energy profile of the beam itself, or the **transverse mode**, is determined by the geometry of the medium, as well as by the geometric optics of the mirrors. If stimulated emission is confined to a volume close to the axis of the resonator, the laser will operate in what is called "transverse electromagnetic mode zero" (TEM_{00}), and the intensity profile will be Gaussian, as described in detail in *UNIT 1.6*. As the effective cross-section of the medium increases, other transverse electromagnetic modes are superimposed on TEM_{00}. These are individually undesirable in lasers designed for use in cytometry because they are, in general, not radially symmetric, but, rather, multilobed, and are likely to produce nonuniform illumination.

In many types of lasers, polarization is introduced into the beam by putting special windows between the medium and the end mirrors. The windows are placed at Brewster's angle to the axis of the system and usually oriented such that light reflected from the surface of the window is directed upward (vertically). At Brewster's angle ($\sim 57^\circ$ for glass), reflection from the window surface is minimized for

vertically polarized light, while a small percentage of horizontally polarized light is reflected out of the cavity. The slight difference in transmissions of the two polarizations is magnified many-fold by the feedback characteristic of the optical resonator structure, with the result that the laser output in a system with such Brewster windows is highly polarized, usually vertically, and typically in a ratio of at least 500:1.

Pump Energy and Efficiency

To produce and maintain the population inversion necessary to sustain stimulated emission and laser action, energy must be injected from the outside. The method by which this **pumping** is done varies with the lasing medium used. In gas, ion, and metal vapor lasers, electromagnetic energy is used to produce and, in some cases, confine the plasma that serves as the lasing medium. In pulsed lasers, in which the lasing medium might be a solution of fluorescent dye or a ruby or yttrium aluminum garnet (YAG) rod, light from a flashlamp is often the pump energy source. In a **continuous wave** (**CW**), as opposed to pulsed, dye, or YAG laser, the output of a **pump laser**, typically an ion laser in the former case and a diode laser in the latter, is used. Diode lasers themselves are pumped by input of electric current. A substantial power density of excitation is typically necessary to produce a population inversion, therefore, for all laser types, there is a threshold level of pump power below which laser action cannot be achieved.

The efficiency of lasers varies greatly. A high-power argon-ion laser emitting a watt or so of light typically consumes ~10 kW of electrical power while in operation; the overall efficiency of this system is therefore ~0.01%. A CW dye laser, optically pumped with the 1-W output of the argon laser, would typically emit a few hundred milliwatts; the efficiency of the dye laser, neglecting the power consumption of the pump laser, is typically 20% to 30%. Diode lasers are also highly efficient. Less efficient lasers are more likely to require forced-air or water cooling, particularly when high-power outputs are needed; this increases their size, complexity, and cost. Efficiency is strongly dependent on the gain of the laser, which may vary substantially for different laser lines. In the example of the argon laser given above, the same power input that could produce 1 W of visible output might be required to produce only 10 to 20 mW of UV output; the efficiency would then drop to between 0.0001% and 0.0002%.

Output Power Regulation and Laser Noise

The ion lasers used in many flow cytometers can be operated in either a **current control mode** or a **light control mode**. In the current control mode, the power supply is regulated to deliver a constant current through the plasma tube, ostensibly producing a constant supply of pump energy. Light output remains constant as long as the mechanical and optical characteristics of the laser do not change substantially during operation. A perturbation such as a slight misalignment of a mirror will, however, decrease light output, even though power supply current remains constant. In the light control mode, a feedback circuit measures light output and maintains it at a nearly constant level by varying the power supply current as necessary. Although this works well when the laser is emitting at a single wavelength, it is more difficult to keep power constant when emission of several lines occurs simultaneously, particularly if the gains differ considerably and/or if there is competition between lines, which occurs when excited molecules in the medium can drop to lower energy states by two or more mechanisms associated with laser emission. The air-cooled argon lasers in benchtop flow cytometers are operated in the light control mode; so are most diode lasers, which are usually built with a light-sensing photodiode in the same package. In the case of diode lasers, the incorporation of a light-control feedback loop into the power supply is necessary to prevent the laser from overheating and self-destructing when it is first turned on. Helium-neon (He-Ne) and Helium-cadmium (He-Cd) lasers typically do not incorporate light control circuits.

Laser noise may originate from several sources. Poor power supply regulation results in light output fluctuations ("light noise") at the frequency of the line current used to run the power supply or at a multiple thereof; when a high frequency switching circuit is used in the power supply, light noise also typically occurs at the switching frequency. In He-Ne and He-Cd lasers, noise may be found at frequencies in the range of a few hundred kilohertz, due either to radio frequency energy used to pump the lasing medium or to fluctuations in the medium itself. In most circumstances, the level of laser noise determines the minimum detectable signal level for scatter measurement channels. Although preamplifier electronics typically remove the steady DC component of the background noise produced in detectors by stray laser light, the AC component of the

background, representing fluctuations around the DC level, is amplified along with the signals produced by particles passing through the illumination beam. To be detectable, the signal from a particle must be substantially above the level of the fluctuations; therefore, a laser with lower light noise allows detection of smaller particles.

Operation in the light control mode does not guarantee protection against noise. If the light output drops substantially enough for the supply current to rise to its maximum value, any further mechanical or optical deterioration cannot be compensated for by either the light control or the current control circuitry; thus, a change in light output will occur. One logical solution to laser noise problems is the incorporation of compensation for changes in source intensity in the signal processing electronics, e.g., amplifiers with gain automatically controlled by a feedback circuit that senses variations in source output at the specific excitation wavelength(s) of interest. Such circuits have been used in several laboratory-built flow cytometers, but are not currently incorporated in commercial instruments.

Laser Peripherals: Harmonic Generation and Modulation

The light produced by lasers, like all other light, has associated electric and magnetic fields, and, because the radiance of a laser beam is substantially higher than that of an incandescent source, the associated electric field intensity may be high enough to produce nonlinear responses in certain materials. One notable application of these phenomena is in **harmonic generation**, in which nonlinear effects in crystals result in generation of light at two or more times the frequency of the incident light. Second harmonic generation, or frequency doubling of the 1064-nm YAG laser line, for example, produces 532 nm, while third harmonic generation, or frequency tripling, produces 355 nm. The same nonlinear crystals may also be used to produce output at the sum and/or difference of the frequencies of two incident beams; in the case of YAG lasers, frequency summing can produce emission at 473 nm. While a reasonably broad range of crystalline materials capable of harmonic generation is available, the range of wavelengths at which continuous (CW) output can be obtained is restricted.

It is sometimes desirable to vary the output power of a laser more rapidly than can be accomplished by adjustments to the power supply. Modulation at frequencies up to several hundred megahertz is possible using electro-optic modulators, which incorporate crystals that change their refractive index as a function of an applied voltage. Acousto-optic modulators, which use sound waves to produce changes in density that affect the light transmission characteristics of a substrate, work at lower frequencies, generally below 100 MHz. The combination of a light sensor and a modulator, connected by a feedback circuit, can be used as a "noise eater," providing the equivalent of light regulated output for a laser into which it was not built.

LASERS USED IN FLOW CYTOMETRY

The first generation of benchtop fluorescence flow cytometers did not offer the user a choice of light sources; they came equipped with air-cooled argon-ion lasers, emitting 10 to 25 mW at 488 nm. Older cell sorters and some modern ones, particularly those in which cells are measured in a stream in air rather than in a cuvette, used and use larger, water-cooled, multiwatt argon- and/or krypton-ion lasers. Various instruments have also used He-Ne, He-Cd, dye, diode, and solid-state lasers, with the latter two types becoming increasingly popular in newer cytometers. While the successful operation of large ion and dye lasers requires some skill and training, fixed-wavelength low-power lasers typically do not allow the end user to make any adjustments beyond adjusting output power. Emission wavelengths of a variety of lasers usable for cytometry are shown in Table 1.9.1.

Argon- and Krypton-Ion Lasers

The lasing medium in an argon- or krypton-ion laser is plasma; when the system is turned on, a high-voltage pulse is used to ionize the gas, allowing it to conduct the relatively high electrical current that maintains the plasma discharge, pumping ionized gas atoms into excited states. Singly ionized argon is capable of several laser transitions at wavelengths ranging from the blue-violet to green (see Table 1.9.1); the highest gain lines are those at 515 and 488 nm, with the 488 nm line predominating in smaller systems and the 515 nm line in larger ones. Doubly ionized argon can undergo laser transitions in the UV, ranging from 275 to 363 nm. The gain of the UV and blue-violet argon lines is relatively low; this means that, while an air-cooled argon laser with a plasma tube on the order of a foot long can be used to produce tens of milliwatts of output at 488 or 515 nm, consuming ~1 kW

Table 1.9.1 Discrete Emission Wavelengths (nm) of a Selection of Lasers Usable for Cytometry[a]

Ar Ion	Kr Ion	He-Cd	He-Ne	Solid state
275.4				
300.3				
302.4				
305.5				
		325.0		
333.6				
	337.4			
				~342 (diode)
	350.7			
351.1				
		354.5		
				355 [Nd:YAG × 3 (pulsed and CW)]
	356.4			
363.8				
				~375 (diode)
				~405 (diode)
	406.7			
	413.1			
	415.4			
				430 (Cr:LiSAF)
				~440 (diode)
		441.6		
454.5				
				457 (Nd:YVO_4 × 2)
457.9				
				460 (semi × 2)
465.8				
	468.0			
472.7				
				473 (summed YAG)
	476.2			
476.5				
	482.5			
488.0				~488 (diode), 488.0 (semi × 2)
				491 (semi × 2)
496.5				
501.7				

continued

Table 1.9.1 Discrete Emission Wavelengths (nm) of a Selection of Lasers Usable for Cytometry[a], *continued*

Ar Ion	Kr Ion	He-Cd	He-Ne	Solid state
514.5				514.5 (Yb:YAG × 2)
	520.8			
528.7				
	530.9			
				532 (Nd:YAG × 2)
		533.7		
				535 (fiber; 515-560)
		537.8		
			543.5	
				560 (fiber), 561 (Nd:YAG × 2)
	568.2			
				580 (fiber)
				592 (fiber)
				593.5 (summed)
			594.1	
			611.9	
			632.8	
				~635 (diode)
		635.5		
		636.0		
	647.1			
				~660 (diode)
				~670 (diode)
	676.4			
	752.5			
				~780 (diode)
	799.3			

[a]Diode, dye, and some solid-state lasers are tunable over ranges of wavelengths. See text for details.

of electrical power, a substantially larger device, typically with a plasma tube 3 or 4 feet long, must be used to generate UV and/or to produce more than a few milliwatts at 457 nm. In the larger ion lasers, a strong magnetic field, generated by a solenoid is used to confine the plasma to the central or bore region of the plasma tube, with the necessary solenoid current further increasing power requirements. It is thus necessary to use water, flowing at a rate of several gallons/minute, to cool the laser. As previously mentioned, the efficiency of argon lasers is low; the optical power output is between 0.0001% and 0.01% of the electrical power input.

The low-power, air-cooled argon lasers in many commercial flow cytometers are almost always equipped with fixed mirrors limiting the emission wavelength to 488 nm. Higher-power air- and water-cooled models usually feature interchangeable mirrors and a Littrow prism assembly that permits wavelength selection. In addition to the strong blue-green and green lines at 488 and 515 nm, argon-ion lasers emit at 454, 457 (violet-blue), 465 (blue), 472, 476 (blue-green), 496, and 501 (green) nm.

Emission can also be obtained in the UV at 351 and 363 nm and in the green at 528 nm using specially coated mirrors; in addition, the largest high-power argon-ion lasers can produce some deep UV lines between 275 and 305 nm.

The plasma tube in an argon laser typically lasts for several thousand hours of operation; replacement thereafter is likely to cost between one-third and one-half the original price of the system. When considering installation of a water-cooled ion laser, the costs of wiring the room with 208- to 220-V, 70- to 100-A, three-phase electrical service, and of providing cooling water via the recirculating cooling system now mandated by environmental legislation almost everywhere, must be factored into the calculations. It is also advisable to verify that the laser will fit, or can be made to fit, onto the cytometer's optical bench.

Large argon-ion lasers can be equipped with special mirrors that permit simultaneous emission at 351/363 nm in the UV and at 488 nm and other visible wavelengths. This provides the dual-wavelength source needed, for example, to do simultaneous analysis of DNA, using the UV-excited blue fluorochrome Hoechst 33342, and surface antigens, using fluoresceinated antibodies. In order to emit in the UV, the laser has to be run at a very high current; since the laser is much more efficient at 488 nm than in the UV, power output at 488 nm is quite high. If the sensor in the light output regulator circuit responds to UV and visible light, or to light at 488 nm alone, there may be considerable fluctuation in UV power output. If the sensor's optical bandwidth is restricted so that 488-nm light is blocked, the sensor and the regulation electronics then respond to fluctuations in UV power output. The relatively large changes in current that may be necessary to keep UV output stable can then result in large fluctuations in power output at 488 nm, which may make it preferable to use separate sources for the UV and the visible.

Krypton-ion lasers first came into use in flow cytometry for two-color immunofluorescence measurements. The 568 nm line from a krypton-ion laser was used to detect antibodies labeled with Texas red or XRITC, both of which are derivatives of rhodamine 101, while 488 nm excitation from an argon laser was used for fluorescein excitation.

Krypton-ion lasers have lower gain and lower efficiency than argon lasers, but emit over a much broader spectral range. They can produce blue-green at 468, 476, and 482 nm, green at 520 and 530 nm, yellow at 568 nm, and red at 647 (the strongest krypton line) and 676 nm, all at once, explaining their popularity for laser light shows. With different mirror sets, krypton lasers can also emit UV (337/350/356 nm), violet (406/413/415 nm), and infrared (752/799 nm) light.

Unfortunately, the optimum values for gas pressure and solenoid magnetic field for krypton laser operation are different for different lines. Since the gains at all lines are low, these parameters must be well controlled to maintain laser action. Krypton-ion laser (and UV argon-ion laser) mirrors need to be extremely clean, and, if the optical alignment of the laser is not near-perfect in the visible, as indicated by maximum all-lines power output near the manufacturer's specs, a user is unlikely to get an ion laser to work in the UV. Low gain at all lines means that krypton laser plasma tubes run hotter and fail sooner than argon laser tubes. There is also substantial competition among the visible krypton lines; for example, running in light control mode with optics that allow simultaneous yellow and red emission often results in alternating fluctuations in the yellow and red power outputs.

Argon- and krypton-ion lasers both normally produce TEM_{00} (Gaussian) output beams. Mixed-gas lasers, filled with argon and krypton and capable of emission at any of the visible output wavelengths available from krypton, as well as at the major argon lines, are also available. These may have multimode output, but can generally be used in flow cytometers because the output beam is not multilobed.

Keeping the optics of an ion laser clean and in alignment is a challenge best met by following the manufacturer's instructions. Mirrors and Brewster windows are generally cleaned with methanol; acetone, which is a good cleaner for some mirrors and destroys others, should be used only if the manufacturer recommends it. Electronic or HPLC-grade solvents are generally free enough of contaminants to be safe to use for cleaning optics, but the slightest contamination of the solvent with grease, from fingers or elsewhere, will result in the deposition of a residue on laser optics, which can drastically reduce output.

Changing mirrors and getting the laser to lase again is a tedious procedure which must be done a little bit differently for each manufacturer's lasers; it is learned by doing, preferably with someone more knowledgeable in attendance, at least the first time, and may remain something of a black art to even experienced operators. Some of the newer large ion lasers

incorporate features that facilitate manipulation of the optics.

The noise characteristics of large and small ion lasers may be different, predominantly due to differences in the types of power supply used. Most older, large ion lasers used linear power supplies, in which most of the noise is at small multiples of the line frequency, typically below 1 kHz. Air-cooled ion lasers use switching power supplies, which have higher noise levels, typically at the switching frequency of a few tens of kHz. The noise on the output of the larger lasers is typically specified as <0.2% RMS, while that on the output of the smaller ones is specified as <1% RMS. The percentage of RMS noise is the coefficient of variation of the output power. As was previously mentioned, laser noise may determine measurement sensitivity, particularly for scatter measurements. This, and not the difference in power output, explains why it is generally possible to detect scatter signals from smaller particles when a larger ion laser is used as a source than when a smaller laser is used in the same instrument.

The complexity of ion lasers and the difference in construction between low- and high-power argon lasers make the relationship between price and power output highly nonlinear. A laser that emits 25 mW at 488 nm costs around $6,000; six or more times that amount buys a laser that delivers anywhere from ten to thirty times the power. If UV output and or multiwatt visible output to pump a dye laser are not essential, a "high-power," air-cooled argon laser, with a power output of over 100 mW at 488 or 515 nm, may be used; these are available for under $10,000, but, in general, solid-state lasers (see below) are now to be preferred to ion lasers.

The first commercial cell sorter, introduced in 1974, incorporated a water-cooled argon laser, capable of emitting several hundred milliwatts at 488 nm, largely because the stream-in-air configuration of the instrument imposed practical limits on the efficiency of fluorescence collection. By the late 1970s, it had become apparent that cytometers incorporating more efficient collection optics and/or cuvettes in which cells were observed could match or exceed the fluorescence measurement sensitivity of the earlier instruments using only a few tens of milliwatts of excitation light, obtainable from low-power, air-cooled argon lasers, which, although still energy-inefficient, were smaller, less expensive, and a great deal more user-friendly than their larger water-cooled siblings. The new millennium brought with it small, energy-efficient (running on standard line current) solid-state lasers emitting tens to hundreds of milliwatts at 488 to 492 nm, which have replaced air-cooled argon lasers in almost all newer instruments and can also replace water-cooled argon-ion lasers when only blue-green light sources are needed. Since 2000, almost all of the excitation wavelengths used in flow cytometry have become available from solid-state sources of one type or another, as can be appreciated from Table 1.9.1.

Helium-Neon Lasers

He-Ne lasers, once the smallest, most efficient, least complicated, least expensive, and most commonly used lasers available, have been displaced from all of the above categories by diode and other solid-state lasers, but remain useful for flow cytometry. The plasma tube of a helium-neon laser is not unlike the tubing of a neon sign; it contains a mixture of helium and neon gases, through which a relatively low electric current is passed, raising helium atoms to electronically excited states. Collisions between the helium and neon atoms transfer the excitation energy to the latter, which may drop to lower excited states via any of several laser transitions, ranging in wavelength from green (543 nm) to infrared (3.39 μm). He-Ne lasers typically require only air-cooling by convection and use fixed mirrors. They are more efficient than argon lasers, yielding 1 to 50 mW optical power for electrical power inputs of a few hundred watts or less.

The most common He-Ne lasers emit red light at 633 nm; other visible wavelengths at which He-Ne lasers are now available include 543, 594, and 611 nm. The head assembly containing a He-Ne plasma tube is typically 1 to 2 feet long; the highest power He-Ne lasers may be twice that length. Almost all He-Ne lasers emit Gaussian (TEM_{00}) beams. Noise levels vary; a 0.8-mW system with 0.05% RMS noise was used for extinction measurements in some commercial systems in the 1970s and 1980s, but the lasers with the >5 mW output generally needed for fluorescence measurement are generally specified for a 1.0% RMS noise level. Red (633-nm) He-Ne lasers used in cytometry are typically polarized. Green (543-nm) systems are most often not polarized, because the extremely low gain of the green line, which limits power output to a few milliwatts, makes it difficult to introduce Brewster windows into the system without unacceptable light losses. Depending on power level, He-Ne lasers cost between a few hundred and a few thousand

dollars; plasma tube lifetimes are typically 10,000 hr or more.

One common application of red He-Ne lasers in fluorescence flow cytometry is in measurements of immunofluorescence, using antibodies conjugated to the phycobiliprotein allophycocyanin. In instruments with reasonably efficient light collection, diode lasers emitting in the 635- to 640-nm region, which are now available at power levels >35 mW, and which are incorporated in some commercial instruments, may be a better choice, although He-Ne lasers are to be preferred when constancy of wavelength is critical (see the discussion of red diode lasers).

Although the 543-nm He-Ne laser wavelength is useful for excitation of immunofluorescence from antibodies labeled with phycoerythrin (one form of which has an absorption maximum at 545 nm), and can also be used to excite DNA stains such as propidium, it is difficult to obtain more than a few milliwatts output at 543 nm. This has made it more practical to use 532-nm frequency-doubled YAG lasers, which are typically more expensive but can produce much more power, in preference to green He-Ne lasers. The 594-nm He-Ne laser can excite Texas red and spectrally similar dyes and combinations of these dyes with allophycocyanin. Although it operates at the same wavelength at which dye lasers are often used for these purposes, the He-Ne laser can produce only a few milliwatts and has never been widely used. Newer, more powerful solid-state devices emitting at or near 594 nm are now available; the major disadvantage of the wavelength is its proximity to the emission region of phycoerythrin.

Helium-Cadmium Lasers

He-Cd lasers, which can emit 5 to 200 mW in the blue-violet (441 nm) and 1 to 100 mW in the UV (1 to 100 mW at 325 nm; 1 to 10 mW at 354 nm), have occasionally been useful sources for flow cytometry. Like He-Ne lasers, they plug into the wall and do not require water-cooling; they need few or no adjustments and are moderately cheaper than most ion lasers. The lasing medium is cadmium vapor; cadmium ions are typically excited by collision with excited helium atoms. The pressure of the cadmium and helium and the temperature of the medium must be carefully controlled to ensure stable operation. He-Cd lasers are intermediate in efficiency between argon-ion and He-Ne lasers. They typically emit TEM_{00} (Gaussian) beams in the blue-violet, and TEM_{01*} (donut-shaped) beams in the UV. Some models are linearly polarized, others are randomly polarized. Plasma tube lifetimes are on the order of a few thousand hours.

The 325- and 354-nm UV lines available from He-Cd lasers are suitable for excitation of the fluorescence of the DNA stains DAPI, DIPI, Hoechst 33342 and 33258, and of the calcium probe indo-1, as well as other UV-excited dyes. Parinaric acid, used for analysis of lipid peroxidation, is optimally excited near 325 nm and only marginally excited at the longer UV wavelengths (350 to 365 nm) available from argon- and krypton-ion and arc lamp sources. The 441-nm He-Cd line is useful for excitation of the chromomycin family of DNA stains (olivomycin, chromomycin A_3, and mithramycin), but these dyes can also be excited by the 457-nm argon-ion laser line and by newer diode lasers emitting at or near 440 nm. He-Cd lasers emitting both 325- and 441-nm are available, with either wavelength selectable by switching filters; there is no significant competition between the UV and blue-violet lines.

The He-Cd medium is also capable of laser transitions in the green and red spectral regions, making it possible to produce a "white-light" laser. This requires a design substantially different from most of those now available, and there are more cost-effective ways of obtaining multiple lines.

The principal problem with He-Cd lasers is noise. The major noise component is plasma noise, at frequencies between 300 and 400 kHz. It is difficult to keep RMS noise levels much below 1.5% even when the laser is new; noise levels tend to increase thereafter, especially if the laser is left idle for long periods of time, because this leads to an irreversible increase in helium pressure in the plasma tube, which increases noise. This effect can be minimized by running the laser for a period of several hours at least once a week. Once the noise develops, however, it cannot be reduced by such regular operation. Noise is not a problem when the laser is used for ratiometric measurement of calcium based on measurement of indo-1 emission in two spectral regions; the noise appears in both the numerator and denominator of the ratio, and is therefore factored out. However, noise can and does interfere with precise measurement of DNA content using DAPI or the Hoechst dyes.

The effects of noise have been reduced in laboratory-built systems using noise compensation electronics and electro-optic modulators; the former, while inexpensive, are

not available in commercial flow cytometers. 325-nm He-Cd lasers are difficult to use in microscope-based instruments because the wavelength is very poorly transmitted through most optical glasses. Unless this wavelength is necessary (as for excitation of parinaric acid), there is no longer much excuse to use a He-Cd laser as a UV source for cytometry, as solid-state and diode sources emitting in the 355- to 395-nm region are now available.

Dye Lasers

Dye lasers are used predominantly as excitation sources for antibody labels based on rhodamine 101, e.g., Texas red and XRITC. They also provide acceptable excitation for the phycobiliproteins phycocyanin and allophycocyanin and their tandem conjugates, and for cyanine dye labels such as Cy5. The dye lasers used in flow cytometry are capable of continuous (i.e., CW) operation; the lasing medium is a fluorescent dye, usually rhodamine 6G, dissolved in an organic solvent such as ethanol or ethylene glycol. Which dye is actually used depends on the wavelengths at which operation is desired; dyes now available for use with blue-green/green (457 to 515 nm) argon-ion pump lasers permit operation at wavelengths extending from 540 to over 900 nm. Selection of the output wavelength for any given dye is usually done with a wedge or filter rather than with a prism.

A dye laser is tunable over a wider continuous range of output wavelengths than is a He-Ne, ion, or He-Cd laser, because the laser transitions in a dye laser are between electronic energy states of a molecule, rather than between electronic energy states of an atom, as in the other types. Each electronic energy state of a molecule has associated with it a number of vibrational and rotational energy states. Excitation of dye molecules by absorption of light from the pump laser typically leaves the molecules in the first electronic excited state and one of the associated vibrational excited states, from which they drop, usually nonradiatively, to the vibrational ground state, producing a population inversion in the presence of a pump power input above threshold. Laser transitions are possible from the first excited electronic/ground vibrational state to any of the vibrational states associated with the electronic ground state. This means that laser action can be sustained over a range of wavelengths, made essentially continuous by the thermal broadening of absorption and emission spectra that is characteristic of all molecules above absolute zero. The dispersion angle of the tuning element determines the precise output wavelength.

The dye in a CW dye laser is circulated through a nozzle, producing a flat-walled stream; circulation is necessary to minimize bleaching and cooling by a heat exchanger. The volume of the lasing medium is quite small, because the gain is very high. CW dye lasers require minimal electrical power, most of which is used to operate the circulator pump. While some dyes bleach faster than others, all need to be replaced after a few months' operation. This, and the facts that CW dye lasers are relatively hard to keep aligned and may not maintain output power as stably as do ion lasers, have restricted their use as sources for flow cytometry.

The threshold power required from the pump laser to achieve output from a rhodamine 6G dye laser is usually ~700 mW; this is generally obtained from a multiwatt, water-cooled argon-ion laser. With 700-mW pump power input, >100 mW of light can typically be obtained from rhodamine 6G at wavelengths selectable between ~570 and 620 nm; although the 14% efficiency of the dye laser is admirable, the pump laser remains extremely inefficient. Dye lasers typically emit Gaussian beams.

For Texas red excitation, a dye laser could be operated at ~595 nm, but a longer wavelength, 605 to 610 nm, is now more often used; this is better for simultaneous excitation of Texas red and allophycocyanin, and interferes less with phycoerythrin emission. The disadvantage of using dye lasers in dual-laser systems lies in the resulting inability to use either the dye laser or the pump laser as a UV source. While, in principle, one can get around this using dual-wavelength UV/visible mirrors in the argon pump laser, this solution requires great technical skill on the part of the user and might also involve frequent plasma tube replacements.

CW dye lasers are now used relatively infrequently in flow cytometry; the few people with enough skill and money to operate and maintain them now seem to be switching to high-power 532-nm YAG lasers (see below) as pumps.

Diode Lasers: Red, Infrared, Violet, and UV

Tens of millions of infrared diode lasers are now sold every year; they are incorporated into compact disc players, laser printers, and

CD-ROM readers, and now cost no more than a few dollars each. Red diode lasers emitting anywhere between 630 and 685 nm are also widely used in laser pointers and bar code readers, and are only slightly more expensive. In recent years, red (635 to 640 nm) diode lasers and, subsequently, violet (~405 nm) and UV diodes, have been incorporated into laboratory-built and commercial flow cytometers, and it is now generally believed that diode and solid-state lasers, which will be discussed in the next section, will almost completely replace other laser types as light sources for flow cytometry.

Like transistors, diode lasers are made of materials classed as semiconductors. The light emission from semiconductors is not from excited atoms or ions, as is the case in ion, He-Ne, and He-Cd lasers, and not, strictly speaking, from excited molecules, as is the case in dye lasers; the electrons that are excited in a diode laser are "free" in a crystalline material. Such free electrons also occur in metals; the nuclei in a metal are packed relatively close together, and the electrons in the outermost shells are not tightly held by any given nucleus, and may be excited from the so-called valence band to the so-called conduction band by ambient thermal energy. Energy transfer among electrons in a metal can occur fairly readily; this is what makes metals good conductors of electricity.

Semiconductors are so named because, while they do not conduct electricity well when in an unperturbed state, they may become conductive in the presence of an applied electric field or of incident light. Their electronic structure differs critically from that of metals in that there is a substantial energy difference, or bandgap, between the valence and conduction bands, with almost all of the electrons lying in the valence band under normal conditions. The application of an electrical current to an appropriately configured semiconductor can result in light emission as electrons relax from the conduction to the valence band; this type of spontaneous emission is what occurs in light-emitting diodes (LEDs).

A laser diode is basically an LED, the geometry of which is tailored to provide a resonator structure that will support stimulated emission. The active regions of diode lasers typically have dimensions on the order of a few micrometers. They use either polished facets on the semiconductor material itself or adjacent structures of differing refractive index to perform the function of the mirrors used in larger lasers. Because the efficiency of diode lasers is extremely high, typically ~20% to 30%, high reflectivity is not needed. Also, because hundreds of lasers can be produced from slices of a single semiconductor wafer, diode lasers are much less expensive than any other type of laser.

The first material of which diode lasers were made is gallium aluminum arsenide (GaAlAs); this is a so-called ternary (made up of three elements) semiconductor. The bandgap energy, and therefore the emission wavelength, is varied by changing the ratio of gallium to aluminum in the semiconductor material. The emission wavelengths theoretically achievable with GaAlAs lasers range from ~650 nm, at which point the material is almost pure AlAs, to ~900 nm, at which point the material is almost pure GaAs. However, the GaAlAs lasers that have been made to date that emit below 750 nm are typically unstable, and succumb to thermal runaway within minutes to hours. The GaAlAs lasers now available in quantity emit at 750 to 780 nm; these are used in compact disc players, CD-ROM readers, and laser printers. Gallium indium phosphide (GaInP) lasers go down to ~670 nm, providing up to 20-mW emission, and aluminum gallium indium phosphide (AlGaInP) devices are now available with a 635-nm emission wavelength and power outputs of 3 to >35 mW. 635-nm diode lasers are now available as light sources in commercial cytometers from a number of manufacturers.

Unlike the other types previously discussed, many diode lasers do not normally emit anything approximating a circular Gaussian beam; their emitting surface is a stripe ~1-μm high and a few micrometers wide. The beam diverges more in the direction perpendicular to the long axis of the emitter surface than in the direction parallel to it. This makes it more difficult to achieve uniform illumination of the illumination volume of a flow cytometer using a diode laser than using lasers that emit Gaussian or near-Gaussian beams. When a spherical or radially symmetric aspheric lens is used to collect light from the laser, the resultant collimated beam is asymmetric, and when focused to a small spot, often shows substantial intensity variations along one dimension or another. Further difficulties are introduced because diode lasers show some lot-to-lot and unit-to-unit variation in beam geometry, simply because of slight differences in structure. Improved beam quality can often be achieved by mounting a small cylindrical or anamorphic lens assembly inside the case of a laser diode, providing a radially symmetric, near-Gaussian output beam that can readily be focused using

the same crossed cylindrical lenses used to derive flow cytometer illumination from other laser types (see *UNIT 1.6*). Also, some newer diode laser configurations also emit beams with profiles quite close to the Gaussian without benefit of such additional optics.

An additional characteristic of diode lasers that differentiates them from most other laser types (dye lasers excepted) is a substantially greater variation in output wavelength, which may vary by as much as several nanometers as a function of small differences in semiconductor composition. Diode lasers are also subject to wavelength variation with changes in temperature.

Output within a desired wavelength range is, at present, best achieved by negotiating with the laser supplier for selected, tested units. In addition to their small size [laser, power regulator, and collimating optics in a package $<$2 in. long and $<$1 in. (2.54 cm) in diameter], minimal power requirements (a 15-mW diode laser draws a fraction of a watt of electrical power), and low cost (a 15 mW, 635-nm diode laser with a circularized beam costs about half as much as a He-Ne laser emitting 15 mW at 633 nm), diode lasers as typically supplied have extremely low noise. A photodiode that senses laser output is built into the case of most laser diodes; this is connected to a feedback-controlled, power supply regulator that converts a nominal 5- to 6-V DC input from a battery or DC supply to the lower voltage required by the diode and regulates current to prevent the laser from self-destructing. The laser effectively operates in light control mode, with $<$0.05% RMS noise; this makes diode lasers well suited for extinction measurements.

The same dyes can be used with 635- to 640-nm diode lasers as with a 633-nm He-Ne laser. The list of dyes usable with 670-nm lasers is somewhat more restricted. These sources are usable for DNA content analysis using rhodamine 800 or oxazine 750, for immunofluorescence analysis using the cyanine label Cy5.5, and for membrane potential analysis using dibenzoDiIC$_1$(5). The latter two dyes are not widely available commercially, nor are flow cytometers with 670-nm lasers. It is relatively difficult and dangerous to build flow cytometers using 780-nm diode laser sources, because it is impossible for most people to see the beam when setting up the laser. Also, although scatter and extinction measurements can readily be done at this wavelength, few dyes suitable for 780-nm excitation have been well characterized for use in cytometry.

Diode lasers are used for optical disc recording and playback, in which applications, achieving a smaller focal spot size allows more information to be stored in and/or retrieved from the same area. Recordable CDs, written and read with ~780-nm diode lasers, top out at ~700 Mb per disc; using a shorter-wavelength (650 to 660 nm) red diode laser instead of an IR laser allows a DVD to store 5.7 Gb on a side. Considerable money and effort have been expended in developing even shorter-wavelength "violet" (~405 nm) diode lasers, which extend optical storage capacities to $>$25 Gb/disc. These lasers are now available with emission in the range from 370 nm (UV) to 445 nm (blue-violet), providing tens of milliwatts of UV and over 100 mW of violet light. They are small and energy-efficient, although they typically require both temperature control and light control to maintain stable output. Within the past two years, laser diodes emitting tens of milliwatts at 488 nm and 450 nm have become available, and a 342-nm laser diode was recently announced.

Violet laser diodes are good excitation sources for a lot of materials that would otherwise require a krypton-ion laser for excitation. The list includes Molecular Probes' labels Cascade Blue and Cascade Yellow, monobromo- and monochlorobimane, both used for detection of intracellular glutathione, CFP, the cyan-fluorescent reporter protein, and the DNA dyes mithramycin and chromomycin A_3. Violet diodes are also very good excitation sources for quantum dots (semiconductor nanocrystals), which, regardless of excitation wavelength, have higher excitation cross-sections as the excitation wavelength decreases. Although shorter (UV) wavelengths would provide even more efficient excitation, violet diodes are currently more cost-effective. Laser diodes operating at the short (370 to 400 nm) end of the UV/violet range are effective excitation sources for DAPI (38% of maximum excitation at 395 nm) and usable with the Hoechst DNA dyes; they cannot be used with the calcium probe indo-1, but it may be possible to synthesize a similar calcium probe that would work with 370-nm excitation. The Hoechst dyes and DAPI can also be excited, albeit suboptimally, by 405-nm laser diodes. By 2002, almost all major manufacturers of fluorescence flow cytometers were offering violet/UV diode lasers as excitation sources.

The major problems encountered with diode lasers in cytometry relate to the variability of their emission wavelengths. It would be prohibitively expensive for a cytometer

manufacturer to specify an acceptable wavelength range of only a few nanometers for diode lasers. As a result, the "635-nm" red diode laser in a flow cytometer might emit anywhere between ~635 and ~645 nm, while the "405-nm" violet diode laser might emit anywhere between ~395 and ~415 nm. If the red laser is used to excite allophycocyanin fluorescence, which will be measured through a bandpass filter with a wavelength of 660 to 670 nm, it is inevitable that the filter will pass more stray laser light if the laser emits at 645 nm than if it emits at 635 nm, making the measurement background higher and thus making it more difficult to resolve cells bearing small amounts of antibody from unstained cells. Red diode lasers, more than other types, also present a second problem due to their spontaneous emission of longer wavelengths than the laser wavelengths; this will also get through the detector filter. The second red diode problem may be solved by placing a filter on the laser to block the spontaneous emission at longer wavelengths; the primary problem can be eliminated by using a 633-nm He-Ne laser instead of a red diode.

In the case of violet diodes, problems typically arise when an attempt is made to detect "side population" (SP) stem cells based on differences in the intensities of blue and red fluorescence emitted by Hoechst 33342 (see *UNIT 9.18*). The intensity of red fluorescence emission is typically very low, even when the dye is excited near its absorption maximum at ~350 nm. Since the excitation cross-section falls off very rapidly >390 nm, a violet laser which, by the "luck of the draw," emits at 415 nm is unlikely to produce a strong enough red signal to permit good discrimination of SP cells, whereas one that emits at 400 nm may be able to do the job. Although the cytometer manufacturers know the precise wavelengths of the lasers they ship with their instruments, this information is not always communicated to the users. The violet diode problem might or might not disappear as increasingly more powerful diodes are incorporated into cytometers; one might also use a dye such as Vybrant DyeCycle Violet (Invitrogen/Molecular Probes; Telford et al., 2007), which can be excited satisfactorily at 415 nm.

Other Solid-State Lasers

In solid-state lasers using media such as ruby and neodymium (Nd)-doped YAG, a rod made from the lasing material is optically pumped. While flashlamps were originally the predominant pump sources for pulsed solid-state lasers, newer, high-power AlGaAs diode lasers, emitting near 800 nm, are increasingly being used for pumping Nd:YAG lasers; this technique, and frequency doubling or summing, have made it possible to produce small, energy-efficient CW solid-state lasers emitting green and blue light.

Laser transitions in Nd can be exploited when this element, nominally in a triply ionized state, is used as a dopant in YAG, yttrium vanadate (YVO_4), or yttrium lithium fluoride crystals, or in glass. The principal laser transition in Nd:YAG is at 1064 nm; slightly different wavelengths are obtained for neodymium in materials other than YAG. The conversion efficiency of Nd:YAG, when optically pumped, may be ~20%, and power outputs of tens of watts may be obtained when a Nd:YAG rod of suitable dimensions is pumped by an array of diode lasers emitting, in the aggregate, close to 100 W at 800 to 850 nm. Efficient frequency doubling can be obtained by placing the doubling crystal inside the laser cavity; power outputs of 5 W or more at 532 nm are now available from Nd:YAG and Nd:YVO_4 lasers, requiring power inputs of less than 100 W from a 110-V AC line. Even high-power doubled Nd:YAG lasers generally require no more than fan cooling; lower-power models may be cooled by convection. Lifetimes are in the thousands of hours, and, although earlier models had noise problems, some current production systems specify <0.1% RMS noise. The output beam is typically Gaussian. Green Nd:YAG lasers with power outputs of 5 to 25 mW, suitable for flow cytometry, may cost between $5,000 and $15,000, depending on the manufacturer. The much less expensive green YAG lasers, now widely available as laser pointers, tend to be too noisy and short-lived for serious use in flow cytometers, but one can get lucky. The large (~5-W) lasers, as was mentioned above, make excellent pumps for dye lasers.

Frequency-doubled, diode-pumped green Nd lasers can be used for excitation of tetramethylrhodamine, phycoerythrin and its tandem conjugates, ethidium, propidium, and 7-aminoactinomycin D, to name a few dyes. Because 532 nm excites much less autofluorescence in mammalian cells than does 488 nm, and is also closer to the excitation maximum of phycoerythrin, the substitution of a 532 nm laser for a 488 nm laser generally makes it possible for a flow cytometer to detect fewer phycoerythrin-labeled or tandem-labeled molecules bound to a cell.

Tripled UV YAG lasers are potentially useful for excitation of DNA stains and calcium probes. They emit at the right wavelength (355 nm), but they typically only operate in pulsed modes. However, a technique called mode-locking allows the lasers to emit regularly spaced pulses at 80 to 100 MHz; this repetition rate is high enough so that the laser behaves more or less as if it were a CW light source. Mode-locked 355-nm Nd:YAG lasers are now available as sources in both benchtop systems and sorters. These lasers are expensive, possibly even more expensive than the UV ion lasers that represent the only alternative sources offering power levels of tens to hundreds of milliwatts, but do share the modest power and cooling requirements of their CW cousins, and therefore do not require the expensive infrastructure needed to support UV ion lasers. A CW 355-nm Nd:YAG laser is expected to be introduced some time in 2009.

An unsuccessful attempt was made some years ago to develop a solid-state CW UV laser using alexandrite, which is beryllium aluminum oxide containing chromium. This material emits between 700 and 850 nm, and can be pumped by 635- to 670-nm diode lasers. UV emission of 10 to 15 mW at ~370 to 380 nm was achieved from a frequency-doubled, diode-pumped alexandrite laser, but stability was an issue.

Alexandrite is an example of a vibronic crystalline laser material. Such materials behave similarly to laser dyes; laser transitions may occur between an excited electronic state and any vibrational state associated with a lower electronic energy state, allowing output to be tuned over a broad range. Titanium-doped sapphire lasers can operate in pulsed or CW mode between 660 and 1180 nm. Pump energy can be supplied by a high-power green YAG laser. Pulsed Ti-sapphire lasers are used in confocal microscopy for multiple-photon excitation of fluorescent dyes and intrinsically fluorescent cellular constituents; their high cost (typically over $100,000) has, to date, limited their use.

High blue-violet power output is available from 457-nm CW lasers made by frequency doubling the output of Nd:YVO_4 . These are fan-cooled and run on house current, drawing ~75 W at the plug, and emit as much as 400 mW. Such lasers can replace the 457-nm water-cooled argon lasers now used in chromosome sorters.

Although green Nd:YAG lasers are good excitation sources for a lot of dyes and labels used in cytometry, they cannot excite fluorescein, which is not only still very popular as a label, but which also has been derivatized into probes for a large number of structural and functional parameters. Fluorescein can be excited at wavelengths as high as 515 nm. The primary emission wavelength of ytterbium YAG (Yb:YAG) lasers is 1029 nm; they could be doubled to 514.5 nm and used for fluorescein excitation. Yb:YAG lasers have some advantages over Nd:YAG in terms of ease of pumping and stability, and this suggests that green Yb:YAG lasers might have fewer problems than green Nd:YAG lasers, but the emergence of 488-nm solid-state lasers (see below) has probably discouraged development of a green Yb:YAG for cytometry, and is also likely to set back development of 473-nm, frequency-summed Nd:YAG lasers, which have been offered in some cytometers.

Doubling a semiconductor laser operating at 750 to 1000 nm will yield UV, violet, blue, or blue-green light. The process is inefficient; a laser capable of producing several hundred milliwatts is required to get a few milliwatts of visible light. The resulting laser system is, typically, considerably more complex and more expensive than a diode laser, because other components, notably, an external mirror and a crystal of the material used for harmonic generation, must be incorporated into the system.

In May, 2000, frequency-doubled, diode-pumped semiconductor lasers emitting 10 mW at 460 nm ± 2 nm and 20 mW or 200 mW models at 488 nm ± 2 nm became available; similar devices are now made by numerous manufacturers and, as was mentioned previously, are incorporated into many benchtop flow cytometers. These lasers are small in size, run on small amounts of house current, and feature long lifetimes (>10,000 hr), but have remained priced in the same range as 488-nm argon ion lasers of equivalent power. The recent introduction of 488-nm laser diodes may eventually lead to lower-priced systems.

A solid-state laser can be made using an appropriately doped glass fiber as the lasing medium. This is an up-conversion rather than a frequency-doubled laser; pumping to the excited state is accomplished by two-photon absorption of light from a diode laser. Fiber lasers can be made to operate at various visible wavelengths; they also have excellent pointing stability, meaning the beam has less tendency to undergo slight changes in direction than do beams from other types of lasers. This would be advantageous for flow cytometry. A variety of wavelengths are now available from fiber lasers (see Table 1.9.1); the yellow and

yellow-orange devices are well-suited for excitation of some of the newer fluorescent protein reporter molecules.

Fibers have also been used to produce "supercontinuum," "white light" lasers, with the coherence and beam characteristics of other laser types, but with simultaneous emission at wavelengths ranging from violet to infrared. Incorporating these lasers into flow cytometers allows wavelength selection to be done with bandpass filters, providing some flexibility (Kapoor et al., 2007), albeit at what is currently a price high enough to discourage widespread adoption.

LASER SAFETY

Lasers that emit more than 800 μW anywhere from the UV to the near IR are classified as hazardous by the U.S. Bureau of Radiological Health. At power levels just above 800 μw, laser beams are potentially harmful to the eye; substantially higher powers, e.g., hundreds of milliwatts in visible and UV beams, can burn skin and set paper and paint, among other things, on fire. Changing or cleaning the optics in some large, water-cooled ion lasers, if it involves removing the outer casing of the laser, may expose the operator to potentially lethal electric currents (tens of amperes at hundreds of volts). To date, the only fatalities associated with laser use appear to have been due to electric shock, and the number of severe eye injuries reported has been relatively small.

Flow cytometers, like other apparatus that incorporate lasers, are required by law to be equipped with light shielding, and operators are required to be provided with safety goggles that prevent laser light from reaching the eye. While the users of most modern benchtop commercial flow cytometers are not generally exposed to the laser beam, people who operate larger instruments, particularly those incorporating large ion lasers with interchangeable mirrors, generally must deal with the laser when the light shields are removed. Unfortunately, adjustments to the laser and illumination optics are best made, and often only possible, when one can see the beam, which requires removing the goggles. In principle, one could use a video camera and/or a laser power meter to determine the presence and intensity of a laser beam while wearing goggles that block the laser wavelength; most people do not.

FUTURE DIRECTIONS

The original version of this unit, published in 1998, suggested that "It would, obviously, be desirable to replace the ion, He-Ne, and He-Cd lasers now used in flow cytometry with diode or solid-state lasers, which would offer greater efficiency, smaller size, lower power consumption, lower noise, and it is to be hoped, lower cost." The newest flow cytometers now offer diode and/or solid-state light sources for UV, violet, blue-violet, blue-green, green, yellow, orange, red, and infrared light, providing adequate excitation for almost all of the fluorescent probes and labels (see *UNIT 1.19*) now in widespread use. Prices for all of these light sources are trending down, which is a good sign.

LITERATURE CITED

Kapoor, V., Subach, F.V., Kozlov, V.G., Grudinin, A., Verkhusha, V.V., and Telford, W.G. 2007. New lasers for flow cytometry: Filling the gaps. *Nat. Methods* 4:678-679.

Telford, W.G., Bradford, J., Godfrey, W., Robey, R.W., and Bates, S.E. 2007. Side population analysis using a violet-excited cell-permeable DNA binding dye. *Stem Cells* 25:1029-1036.

KEY REFERENCES

Harbison, J.P. and Nahory, R.E. 1997. Lasers: Harnessing the Atom's Light. Scientific American Library, New York.

Aimed at the interested layman, this book is beautifully illustrated and includes a detailed discussion of the operation of semiconductor lasers.

Hecht, J. 1992. The Laser Guidebook, 2nd ed. Tab Books (McGraw-Hill), Blue Ridge Summit, Pa.

Hecht, J. 2008. Understanding Lasers: An Entry-Level Guide, 3rd ed. Wiley-IEEE Press, New York.

Hecht's books provide substantial technical detail about lasers in a manner accessible to readers without a strong background in physics and engineering.

Kapoor et al., 2007. See above.

First description of the use of a supercontinuum laser in flow cytometry.

Shapiro, H.M. 2003. Practical Flow Cytometry, 4th ed. Wiley-Liss, Hoboken, N.J.

This book provides more particulars about specific flow cytometric applications of various lasers than appear here.