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Near infrared lasers in flow cytometry

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ABSTRACT

Technology development in flow cytometry has closely tracked laser technology, the light source that flow cytometers almost exclusively use to excite fluorescent probes. The original flow cytometers from the 1970s and 1980s used large water-cooled lasers to produce only one or two laser lines at a time. Modern cytometers can take advantage of the revolution in solid state laser technology to use almost any laser wavelength ranging from the ultraviolet to the near infrared. Commercial cytometers can now be equipped with many small solid state lasers, providing almost any wavelength needed for cellular analysis.

Flow cytometers are now equipped to analyze 20 or more fluorescent probes simultaneously, requiring multiple laser wavelengths. Instrument developers are now trying to increase this number by designing fluorescent probes that can be excited by laser wavelength at the "edges" of the visible light range, in the near ultraviolet and near-infrared region. A variety of fluorescent probes have been developed that excite with violet and long wavelength ultraviolet light; however, the near-infrared range (660–800 nm) has yet seen only exploitation in flow cytometry. Fortunately, near-infrared laser diodes and other solid state laser technologies appropriate for flow cytometry have been in existence for some time, and can be readily incorporated into flow cytometers to accelerate fluorescent probe development. The near infrared region represents one of the last "frontiers" to maximize the number of fluorescent probes that can be analyzed by flow cytometry. In addition, near infrared fluorescent probes used in biomedical tracking and imaging could also be employed for flow cytometry with the correct laser wavelengths. This review describes the available technology, including lasers, fluorescent probes and detector technology optimal for near infrared signal detection.

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1. Introduction

Flow cytometry has become a critical and nearly ubiquitous technology in the field of cell biology, particularly in the biomedical sciences. Flow cytometers permit the analysis of very large numbers of single cells using lasers to excite cell-associated fluorescent probes and filter and detector combinations to detect the often low levels of fluorescence associated with these markers. The original flow cytometers from over forty years ago could only detect one or two fluorescent markers using a single laser source [1]. Modern cytometers now employ multiple laser sources simultaneous to excite a vast array of fluorescent probes, with the simultaneous detection of fifteen or more extracellular or intracellular proteins becoming routine [2]. The wide variety of laser wavelengths now available from small diode-pumped solid state (DPSS) or direct laser diodes means that virtually any biological fluorescent fluorescent probe with a visible light excitation and emission spectra can be analyzed by flow cytometry, if the correct laser wavelength is available. Commercial cytometer manufacturers have taken full advantage of modern laser technology to provide a wide variety of laser wavelengths for single cell analysis [3,4].

The earliest flow cytometers developed in the 1950s and 1960s required an intense light source both to measure cellular light scattering, and to excite the first fluorescent probes. The first cytometers uses mercury arc lamps, capable of producing much brighter and spectrally distinct light that that available from other lamp sources. However, development of the earliest flow cytometers coincided fortuitously with the development of the first lasers, which also become a practical technology in the 1960s. Most of

Abbreviations: AF647, Alexa Fluor 647; AF660, Alexa Fluor 660; AF700, Alexa Fluor 700; AF750, Alexa Fluor 750; AF790, Alexa Fluor 790; APC, allophycocyanin; APD, avalanche photodiode; Cy, cyanin; HeNe, helium-neon; NIR, near infrared; PE, phycoerythrin.

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the earliest cytometers used water-cooled argon and krypton ion lasers as their primary light sources [3,4]. The traditional blue-green 488 nm laser line produced by argon-ion sources was and remains (via newer solid state sources) the primary laser line for most flow cytometric analysis. The 488 nm line excites fluorescein, a low molecular weight fluorochrome that forms the molecular basis for a wide variety of fluorescent cell tags and physiological probes. The cyanobacterial energy exchange protein phycoerythrin (PE) and its tandem dyes, including PE-Texas Red, PE-Cy5 and PE-Cy7, are also excited at 488 nm, and could be distinguished spectrally from fluorescein using bandpass filters, dichroic mirrors and electronic compensation of spectral overlap. These technologies allowed multicolor analysis of multiple cell surface markers. By combining fluorescein, PE and the PE tandem dyes, up to six cell markers could be analyzed simultaneously.

While the blue-green 488 nm line was the primary excitation wavelength used for flow cytometry, the other laser lines produced by argon-ion and krypton-ion lasers were also employed [3,4]. Gas lasers can produce ultraviolet, violet, blue, green, yellow and red laser lines, and all were used to excite specialized fluorochromes in the early days of flow cytometry. The ultraviolet lines produced by argon and krypton lasers could excite dyes like the DNA binding probes DAPI and Hoechst 33342 and 33258, and the green and yellow lines from krypton sources could excite rhodamine and sulforhodamine based fluorescent probes [5]. The long red 641 and 647 nm lines produced by krypton-ion lasers were used to excite newly available red-excited phycobiliproteins like allophycocyanin (APC), and the monomeric cyanin dyes Cy5 and Cy7. These dyes could also be excited with helium-neon (HeNe) lasers emitting at 633 nm, which were air-cooled and easier to use [6,7]. However, the large size, high cost and heavy maintenance requirements of these water-cooled laser sources made cytometry with more than one laser source difficult.

This changed with the advent of laser diodes in the late 1980s. These small, air-cooled and relatively inexpensive lasers allowed the easy incorporation of additional lasers into cytometers already equipped with a primary blue-green source [8,9]. The first laser diodes emitted in the near-infrared and long red range, from approximately 660 nm to above 1200 nm. Laser diodes emitting at approximately 640 nm were introduced into flow cytometers in the early 1990s, as replacements for krypton-ion and HeNe sources for the excitation of APC and Cy5 [9]. Small HeNe lasers continued to be used in flow cytometers for some time, but red laser diodes became the dominant second laser source and remain so today. A red laser diode can excite APC, its tandem dyes (APC-Cy5.5 and APC-Cy7) as well as other low molecular weight dyes like Alexa Fluor 700, allowing up to three fluorescent probes to be added to the probes excited at 488 nm. Adding this second laser source allowed eight to nine fluorescent markers to be analyzed simultaneously.

Violet laser diodes, based on different semiconductor chemistry than red and near infrared diodes, were the next laser type to see wide usage in flow cytometry. These modules excite in the 395–415 nm range, just within the visible spectrum [10]. Originally developed in the mid-1990s, they were first incorporated into flow cytometers in the late 1990s by Shapiro and colleagues [11]. The water-cooled krypton-ion lasers used early in flow cytometry could also produce violet lines at 407, 413 and 415 nm, motivating the development of several fluorescent probes that could be excited in this range. Cascade Blue and Pacific Blue were two coumarin-based low molecular weight fluorescent probes that excited in this range and emitted in the blue 450–480 nm range. The incorporation of small violet laser diodes into cytometers as a third source revived the use of these dyes, and spurred the development of additional fluorescent probes that could take advantage of this laser wavelength [12,13]. Quantum dots or nanocrystals were developed in a form that could be conjugated to antibodies; a series of quantum dots ranging in emission from 525 to 800 nm (Qdot 525, 585, 605, 655, 705 and 800) were initially used with violet laser excitation [14]. More recently, the Brilliant Violet dyes (Sirigen) have been developed, a group of polymer based fluorescent probes that can be "built" with specific excitation and emission characteristics. The Brilliant Violet series (including BV412, BV510, BV570, BV605, BV650, BV705 and BV787) can be easily conjugated to antibodies, and can be used simultaneously to add seven additional fluorescent parameters to the probes already excited by blue-green and red lasers [15]. A cytometer now equipped with blue-green, red and violet lasers could now excite up to sixteen fluorescent probes simultaneously, a substantial number. Many commercial cytometers are now equipped with these three laser wavelengths.

Other laser wavelengths from modern solid state lasers have also been incorporated into flow cytometry. Green and yellow lasers, including 532, 552 and 561 nm, are now common fixtures on cytometers. These laser wavelengths provide more efficient excitation of phycoerythrin and its tandems, and allow better excitation of red fluorescent proteins like DSred and mCherry, which are poorly excited at 488 nm [16–18]. However, these useful wavelengths, while giving improved excitation of many fluorescent probes, have not increased the total number of *simultaneous* parameters available for flow cytometry. With red and violet laser used to their maximum utility, the search was on to look for additional excitation wavelengths and fluorescent probes that could add to the existing array of simultaneous markers. Ultraviolet (UV) lasers have been used in flow cytometry (albeit on a limited basis) since its inception, being originally generated by argon and krypton sources. They were primarily used to excite DNA binding dyes like DAPI and the Hoechst dyes, and to excite the calcium flux probe indo-1 [5]. UV excited molecules suitable for conjugation to antibodies for cell surface labeling, including the coumarin dyes, were not bright, and had to contend with considerable amounts of autofluorescence generated by UV excitation [4]. The primary UV excited fluorochrome aminomethylcoumarin (AMCA) has seen little use in flow cytometry for this reason. This situation changed with the development of the Brilliant Ultraviolet (BUV) series, similar in principle and structure to the Brilliant Ultraviolet dyes. These probes excite well at 355 nm, and emit at uniform wavelengths from the violet to the red. At the time of this writing, BUV395, BUV496 and BUV737 were available. These probes are not well excited by violet laser excitation, and conversely the Brilliant Violet dyes are not well excited by UV. Their emission wavelengths have been staggered with the BV dyes, permitting even better spectral separation. Like the BV dyes, the BUV dyes will add an additional group of parameters for high-dimensional flow cytometry for cytometers equipped with UV lasers. UV lasers remain expensive fixtures on cytometers, but the recent development of small solid state units will make their incorporation into flow cytometers much more feasible.

At this point, the near UV, violet and most visible wavelengths have been extensively "tapped" as excitation sources for flow cytometry, both for the excitation of novel fluorochromes, and to increase the total number of simultaneous fluorochromes that can be used for high-dimensional cell analysis. The near-infrared (NIR) laser range is the only remaining area of the spectrum that has not been extensively investigated for flow cytometry. NIR laser diodes based on InGaP, AlGaInP and GaAs semiconductor chemistry were some of the earliest laser diodes developed (Fig. 1), so laser sources in this range applicable for flow cytometry (ranging from 660 nm to 800 nm) are available. A growing number of fluorescent probes are also available; Cy5.5, Cy7, Cy7.5 and their derivatized counterparts Alexa Fluor 680, 700, 750 and 790 are available in chemical forms appropriate for protein conjugation