

Flow Cytometry: OPSL Technology Meets Critical Needs

Compact platform supports the trend to more excitation wavelengths

●▶ Flow cytometry is a widely used tool for counting and/or sorting cells in both clinical and research medical applications, as well as animal husbandry and testing of genetically modified organisms. One important cytometry trend is the use of more laser wavelengths in order to simultaneously and optimally excite a higher number of fluorescent labels. This, in turn, allows counting or sorting on the basis of a larger number of parameters in a single instrument, which reduces overall measurement time and cost.

In the past, multiple wavelengths were often provided by gas lasers or multiple conventional solid-state lasers, but each of these technologies has both significant practical and cost drawbacks for cytometry. Fortunately, optically pumped semiconductor lasers (OPSLs) successfully address these drawbacks, and also provide a simple route to a greater number of wavelengths than previously available.

Flow Cytometry Background

Flow cytometry uses laser excited fluorescence signals to count and/or sort fast flowing cells according to one or more parameters. These can be cell-surface properties, i.e. the presence of specific surface antigens, which are used for example as unique identifiers of white blood cell type. Here a concentrated blood sample is treated with commercial fluorescent reagents which selectively bind to these surface antigens, so that different cells are tagged to produce a different fluorescent signal. This is the basis of the commonly used complete blood count (CBC).

In an application called flow karyotyping, cells may be differentiated according to nuclear characteristics. Here the DNA is fluorescently labeled and cells sorted based on the amount of DNA they contain. This enables triploid cells and diploid cells to be easily distinguished, which is of interest for

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genetically modified animals. It also allows screening for chromosomal abnormalities as in fetal testing for Down's syndrome.

More recently, cells can be screened according to nuclear activity rather than just nuclear contents, using so-called reporter genes that produce green fluorescence protein (GFP) or related fluorescent proteins. This can be used to test for elevated transcription/translation activity of target genes. And even more subtly, it can be used to track promoter gene activity, where the gene for a fluorescent protein such as DsRed gene is inserted into the cell's DNA at a locus under the control of the promoter gene of interest.

Figure 1 schematically illustrates the basic working principles of flow cytometry. After the cell population has been labeled in one of the ways just described, they are loaded into the instrument, suspended in buffer. They are then forced through a nar-

row orifice, wide enough to pass only one cell at a time. This single-file stream of cells is intersected by the focused laser beam(s). Resultant fluorescence and/or scattered light is captured by receiver optics that pass the light through bandpass filters on to one or more photodetectors. (Some high end instruments now use a linear CCD array in conjunction with a dispersive optic). The instrument software compares the intensity of the fluorescence signals in the various wavelength bands defined by the filters. This enables the instrument to assign the type of each passing cell. In counting applications such as CBC, the instrument merely logs and archives the accumulated counts. In sorting applications, target cell types are deflected by a transient electric field into one or more collection tubes.

In terms of the laser, key requirements of flow cytometry include good spatial mode quality, low noise and high pointing

stability. On the practical side, long lifetime, high reliability, good unit-to-unit consistency and low cost of ownership are also advantageous, especially to the OEM.

Meeting the Challenge of Multiple Wavelengths

A single laser wavelength, most commonly at 488 nm, can be used to excite two or more fluorophores whose fluorescence emission spectra can be confidently resolved in this way. But further increasing the number of fluorophores that can be simultaneously scanned requires using multiple laser wavelengths, each exciting one or more different fluorophores. An established method of incorporating multiple wavelengths is to use a multi-line ion laser, either krypton ion or more commonly an argon ion laser. Generally, the krypton ion offers more wavelengths, particularly in the red, where an argon laser has no output line. But the argon laser has the advantage of higher power and often longer tube lifetime. With both types, there can be quite a large difference in intensity between the various emission wavelengths; typical argon ion lasers deliver most of their power in just two lines at 488 and 514 nm. For this reason, the cytometer also includes an acousto-optic tunable filter (AOTF) in front of the laser, in order to selectively attenuate different wavelengths and thereby produce a better intensity balance. To cover for the lack of red wavelengths when using an argon ion laser, a HeNe (at 633 nm) – or more recently a red diode laser – is also used.

To both OEMs and end users, gas lasers are increasingly viewed as dinosaurs. Specifically, ion lasers are bulky, power hungry, and extremely inefficient, requiring water cooling and wasting most of their input electrical power as heat, all of which adds up to high cost of ownership. Plus their performance drifts with time, often requiring optical tweaking to maintain optimum performance. HeNe lasers are reliable but only produce a single wavelength and are limited to very lower power.

Nonetheless, these gas lasers have continued to enjoy success in flow cytometry, in spite of the solid-state laser revolution that has characterized most other areas of bioinstrumentation. This is a result of the limited wavelength availability, the high capital cost of some early solid-state alternatives, and the fact that solid-state lasers typically are single-wavelength devices. So if a cytometry application needs multiple wavelengths, it needs multiple solid-state lasers. In addition to the cost of multiple lasers, there is also

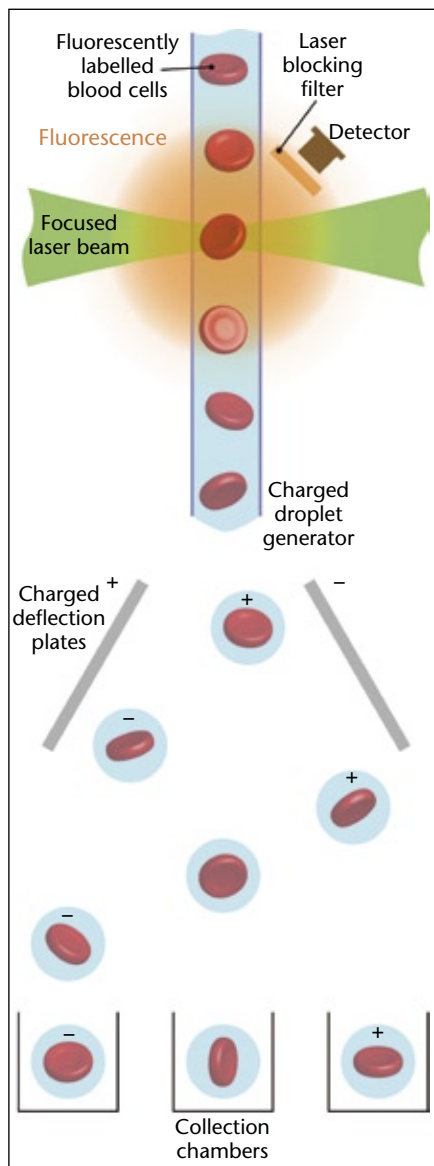


FIGURE 1: In flow cytometry, fluorescently labeled cells flow in single file through an interaction zone where they are illuminated by focused laser beam(s). The fluorescence is split into wavelength bands and measured by photodetectors. In sorting applications, an electric field diverts the cells into collection tubes.

the challenge of packaging these lasers and their beam delivery optics within the confines of the instrument.

OPSL Background

OPSL technology now successfully addresses all of these problems, and, in fact, was developed specifically to address both the output and practical limitations of ion and other laser technologies for life sciences and related applications. This is accomplished because OPSLs are based entirely on semiconductor and solid-state technology that is much more energy efficient than gas lasers.

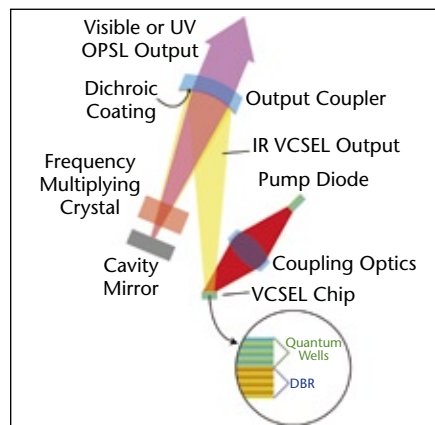


FIGURE 2: In a OPSL, a laser diode pumps a semiconductor chip containing quantum wells. The emission wavelength can be pre-selected over a wide wavelength range simply by changing the stoichiometry and dimensions of these quantum wells.

TRUE CW UV

UV excitation at 355 nm is increasingly used for both cell counting and cell sorting. Frequency-tripled CW lasers based on DPSS are not well suited to this application. Multi-mode lasers with intracavity tripling have too much amplitude noise – because of the “green noise” problem which is magnified for frequency tripling, versus frequency doubling. Green noise can be eliminated using a single-mode approach, but this is not cost-effective for flow cytometry, particularly for OEM applications. So these applications have continued to use ion lasers or mode-locked DPSS lasers which have sufficiently high peak power for extra-cavity doubling. However, the short pulse duration and high peak power of these quasi-CW lasers is a double-edged sword, with the increased potential to cause damage to DNA when tightly focused in a cytometer (or microscope for that matter). Fortunately, the near-zero excited state lifetime of the OPSL gain medium means that green noise is completely absent in these lasers. So a frequency-tripled CW OPSL at 355 nm offers the cost advantages and simplicity of multiple longitudinal mode operation while providing noise characteristics that are equal to or better than a single mode-DPSS. And most importantly, the output is truly CW with no potential for photodamage issues due to high peak power. The Coherent Genesis 355 series ultraviolet lasers have been available for about 12 months and not surprisingly have displaced earlier technologies at most flow cytometer manufacturers, even in that short time period.



FIGURE 3: The inherent simplicity of the technology means a low-noise OPSL can be incredibly compact as in this breadboard example.

In an OPSL, the gain medium consists of a large area, semiconductor VCSEL (vertical cavity surface emitting laser). This is a monolithic III-V semiconductor chip which contains layers of quantum wells specifically designed to efficiently absorb pump radiation and re-emit laser light. Beneath these quantum wells is another semiconductor structure that acts as a low-loss DBR (Distributed Bragg Reflector) mirror optimized for the specific OPSL output wavelength.

Pump light is supplied to the VCSEL by one or more semiconductor diode lasers or diode laser arrays. Infrared laser output is produced which reflects off a dichroic coating on the output coupler and then goes through a frequency multiplying crystal to the rear cavity mirror. The visible or ultraviolet wavelength light produced in the non-linear crystal passes through the dichroic coating on the output coupler and exits the laser cavity.

The output wavelength of the OPSL is determined by the characteristics of the quantum wells in the VCSEL structure, and can be optimized for any wavelength over a broad spectral range in the near-infrared. Efficient intracavity frequency multiplication (e.g. SHG, THG) then enables the final output to be set anywhere throughout the visible spectrum, as well as in the ultraviolet. In commercial OPSLs, a narrowband birefringent filter (BRF) is used to narrow the output and hold its wavelength at the peak of the SHG curve. This BRF limits the output to between three and 10 stable longitudinal modes. Unlike other solid-state lasers, the very short excited state lifetime for the OPS quantum wells eliminates dynamic power oscillation between these modes, resulting in frequency doubled or tripled output characterized by low noise – yet without the cost and complexity of resorting to single-mode operation.

Additionally, OPSL output power can be raised simply by increasing the amount of pump light, without any issues of thermal lensing in the thin rear-cooled gain chip.

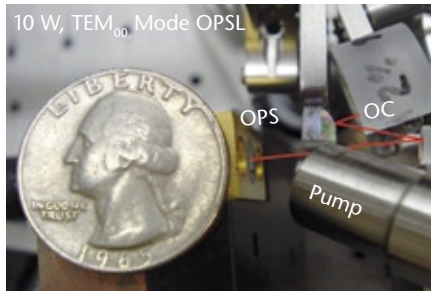


FIGURE 4: The simplicity of OPSL enables over 200 mW from a palm-sized package.

THE COMPANY

Coherent

Since its foundation as a laser manufacturer in 1966, Coherent Inc. has become the technology and market leader in a number of areas. The company, which is headquartered in California, has R&D and manufacturing facilities around the world – in Europe these are in Germany and Great Britain. A global service and sales network supports customers in industry, medicine and science.

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Thus, OPSL technology provides scalability in terms of both wavelength and power, making it a highly future-proofed platform.

OPSL Advantages for Flow Cytometry


The inherent simplicity of the technology means a low-noise OPSL can be incredibly compact as shown in the breadboard example in figure 3. So the final laser can literally sit in the palm of the hand (see Figure 4) with no need for external cooling water fans or cooled baseplates. This allows multiple lasers to be incorporated within an instrument and/or enables the laser to be mounted closer to the interaction zone, minimizing the cost of beam delivery optics.

Just as important, OPSL technology is very cost-competitive for OEMs. That's because alignment of the cavity is not critical, enabling assembly using semi-robotic methods, and providing economy of scale. Other cost savings come from the relaxed pumping wavelength requirements, which eliminates the need and yield issues of se-



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lecting diodes whose emission falls in a very narrow wavelength band. Moreover, no feedback loop is necessary to tightly control the diode operating temperature and hence wavelength. This aspect contributes to other advantages of OPSLs, namely their low power and thermal budgets. And finally, extensive surveying of over 20,000 OPSLs now in the field shows that this highly reliable technology provides lower cost of ownership than any other solid-state visible laser technology to date.

Together these advantages have enabled the development of cost-effective flow cytometers with multiple excitation wavelengths, all from solid-state sources.

Wavelengths – Old and New

In terms of wavelength, OPSL is unique in its wavelength selectability, which allows them to be made for almost any wavelength from the near UV through near-IR. In a mature application like flow cytometry, output wavelengths can therefore be divided into “legacy” wavelengths and new wavelengths. The former refers to well-established wavelengths, previously available from older laser technologies. Standout examples of these are blue wavelengths of 460 and 488 nm, which are a direct replacement for argon ion output at 458 and 488 nm. Indeed, the 488 nm wavelength is the most widely used throughout bioinstrumentation applications that rely on laser-excited fluorescence and over 15,000 OPSLs are in the field at this wavelength alone. At present, output powers range from 10 mW to 500 mW.

Of course the other strong argon ion line is in the green at 514 nm. While it has been less widely used than 488 nm, this is a wavelength of growing importance for multi-wavelength excitation, and OPSLs at this wavelength were launched at Laser Munich 2009. Another green legacy wavelength is 532 nm, traditionally obtained from frequency-doubled DPSS lasers. The availability of low-noise OPSLs at this wavelength using the same platform is now enabling cost-effective integration of this wavelength into the cytometer laser arsenal.

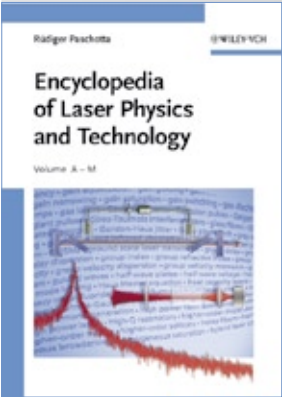
But one of the unique and strategically important advantages of OPSLs is their arbitrary wavelength selectability. For cytometry and indeed any bioinstrumentation application this represents a paradigm shift where the laser is designed to fit the optimum needs of the application, instead of trying to fit the application to available laser wavelengths.

The first “new” wavelength developed for flow cytometry is 560 nm. The main purpose of this wavelength is optimized excitation of DsRed, commonly attached to reporter genes. DsRed has been previously used in flow cytometry using the “nearest fit” 568 nm output line of the krypton ion laser. Obviously, there are many other fluorescent labels used in bioinstrumentation (e.g. the Alex Fluor series) that could now be applied to flow cytometry, supported by fully optimized laser wavelengths. Moreover, as new fluorescent proteins such as the mRFP series are further discovered and manipulated, these too can be used in flow cytometry, with lasers that will exactly match their excitation wavelength requirements.

Conclusion

Flow cytometry is rapidly evolving beyond just counting and sorting by one or two surface antigens. Newer applications are often best supported by using multiple excitation wavelengths, many beyond the restricted portfolio available from legacy lasers. OPSLs are turning out to be near-perfect lasers to support this trend, providing the ideal combination of beam quality, compact packaging, high reliability, cost-effective manufacturing and low noise performance.





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This authoritative two-volume encyclopedia helps to master the large variety of physical phenomena and technological aspects involved in laser technology and the wider field of photonics. Besides explaining in detail the physical principles and common techniques of laser operation, it also addresses such supplementary topics as ultrashort pulses, optical communications, optoelectronics, general optics, and quantum optics. References to selected scientific articles and textbooks aid readers in their further studies, and the cross-disciplinary approach makes this four-color encyclopedia of huge benefit to a wide audience in industry, government, and academic research.

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