

# New Laser Tools Benefit Confocal and Multiphoton Microscopy

By Darryl McCoy

**N**ew wavelengths, flexible multi-laser systems and higher power are key trends in CW lasers for confocal instruments, while new compact economical femtosecond lasers will benefit both OEMs and end users in multiphoton imaging.

## Economical Compact Femtosecond Lasers

Multiphoton microscopy techniques (e.g., two-photon excitation, three-photon excitation, SHG, SRS, CARS) offer numerous advantages over other optical microscopy methods, including inherent three-dimensional imaging. Moreover, because these techniques typically involve minimal to no photo-damage, they are compatible with live tissue imaging over extended time intervals. And, since near-IR laser wavelengths are employed, multiphoton microscopy is capable of deep tissue imaging, which is vital in fields like neuroscience where researchers want to interrogate processes deeper in the murine brain. In spite of these well-known advantages, multiphoton microscopes lag far behind confocal microscopes in overall sales, installations and penetration into pre-clinical applications. A contributory reason for this is cited as the higher cost of ownership and size of tunable femtosecond sources compared to visible lasers.

Laser manufacturers have been focused on this challenge for some time and we are now starting to see dramatic improvements in all three of these parameters via a new generation of femtosecond lasers that are highly optimized for multiphoton microscopy. An example is the new Axon suite of lasers from Coherent. These are fixed single wavelength sources designed to achieve a breakthrough cost point. Just as important, they provide the high (up to 1 Watt) average power, fast (80 MHz) repetition rate, short (<150 fs) pulse width and internal GVD pre-compensation needed for demanding imaging tasks. These are compact, air-cooled sources with a smaller footprint than the typical laptop computer. Moreover, they are available with integrated fast power control and internal modulation so the laser can now be attached directly to the side of the microscope scan head, potentially negating the need for an optical table.



*Figure 1: Darryl McCoy presents at LASER show in Munich 2019 a new generation of compact and cost-conscious ultrafast lasers – Axon Series – is poised to lower some of the hurdles to more widespread adoption of multiphoton microscopes.*

The first two models are offered at 920 nm and 1064 nm. The 920 nm is ideal for GFP and its derivatives and for short wavelength  $\text{Ca}^{2+}$  indicators such as GCAMP. The 1064 nm is an excellent match for both red shifted  $\text{Ca}^{2+}$  indicators and red fluorescent proteins. Other wavelengths are expected soon, all with identical form, fit and function.

## New NIR Wavelengths for Upconversion of Nanoparticles

In fluorescence microscopy, and particularly confocal microscopy, researchers continue to extend the flexibility and range of these techniques by developing new probes, dyes and fluorescent proteins – often at new wavelengths. Compact continuous wave (CW) lasers for confocal microscopy utilize two complementary laser technologies in identical compact packaging: optically pumped semiconductor laser (OPSL) and laser diode. Fortunately, both of these are wavelength scalable, so when a new wavelength is needed, laser manufacturers like Coherent can quickly respond with lasers with perfectly matched wavelengths.

An example of this is the recent growing interest in using upconversion in rare earth (lanthanide) doped nanoparticles. Upconversion of light results in anti-Stokes shifted signals. Most upconversion mechanisms rely on highly non-linear optical effects and therefore require a pulsed laser with high peak power. But lanthanide ions such as  $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$  and  $\text{Ho}^{3+}$  have a ladder of excited stationary states that enables highly excited (visible and UV emitting) fluorescent states to be reached using low power CW near-IR lasers, eliminating the chance of autofluorescence background. The nanoparticles are often co-doped with  $\text{Yb}^{3+}$  that very efficiently absorbs at 980 nm and excites the other ions via energy transfer. Because the excitations do not include the outermost valence electrons, these fluorophores are resistant to photobleaching. They also have a long (millisecond) radiative lifetime enabling interesting time-dependent studies. Plus, the long excitation wavelength allows deeper imaging due to reduced scatter losses. Furthermore, because each nanoparticle can be doped with multiple ions, they don't "blink" like other single-site fluorophores.

To excite these new fluorophores optimally, Coherent specifically developed new members of the OBIS family of lasers with output wavelengths at 785 nm, 808 nm, and 980 nm. These are smart lasers where the laser driver and optics are integrated in a single compact package with identical form, fit and function at all wavelengths to enable plug-and-play functionality.

### Higher Power for Superresolution Techniques

Researchers in numerous areas of biology are increasingly looking to correlate single molecular agents, proteins and transmitters with macroscopic behavior. Fluorescence microscopy is obviously a key tool in these studies because of its unique ability to map specific molecules within a cell. However, one limitation of optical microscopy has been the diffraction limit, where the spatial resolution of a microscope could not be smaller than half the wavelength of the interrogating light.

The diffraction limit was eventually breached by the development of so-called superresolution microscopy techniques with resolution down to a few nanometers – see figure 2. Examples such as STORM and iPALM rely on random stochastic switching, as pioneered by Eric Betzig and William Moerner. Techniques like STED and RESOLFT rely on using a second laser beam to deterministically switch probes in a pre-defined pattern, developed by Stefan Hell. (Hell, Betzig and Moerner shared the 2014 Nobel Chemistry prize for this work.)

All these superresolution techniques need higher (and variable) power since they depend on switching fluorophores on and off via bleaching or saturation. Until recently, this meant using available powers in the watts range. But, this represents power and cost

overkill and unnecessarily increases the probability of sample photobleaching. The optimum power is actually a few hundred milliwatts and laser manufacturers now support these applications with a growing number of laser wavelengths available in the 250–500 mW power range. Examples of this type of power-optimized laser include the Sapphire series from Coherent, where the power can be smoothly varied with no impact on beam output properties.

### Wavelength Flexibility – Simplified Integration

For both users and builders of fluorescent (confocal) microscopes, a widely used keyword today is “flexibility,” primarily referring to the ability to have a very broad palette of wavelengths attached to the microscope. This must be implemented in a manner that allows researchers to use multiple wavelengths simultaneously or switch between different wavelengths nearly instantaneously with pushbutton (software) simplicity. The only simple way to achieve this is to use multiple fiber coupled lasers where each laser is coupled into a single-mode, polarization preserving fiber to maximize the beam quality at the microscope scan head. Coupling a laser into this type of fiber can take many hours of skilled labor, which is why manufacturers like Coherent offer a full range of lasers with a permanent (pigtail) fiber attached. The other challenge is how to couple all the different laser fibers into one or two microscope ports. Coherent developed OBIS Galaxy as a novel solution to this problem.

OBIS Galaxy uses refractive optics to connect up to eight different input wavelengths into a single output fiber, with complete plug-and-play simplicity. Importantly, it preserves single-mode output and linear beam polariza-

tion, requires no mechanical adjustments, and is completely passive, i.e. does not use any electronics. And, like the ubiquitous USB in electronics, this patented (U.S. patent number 8599487) revolutionary module is designed to function as an open-access architecture using standard available fiber connectors (FC/UFC), so that it can be used with any fiber-coupled laser that meets the requisite performance levels in the areas of beam quality and wavelength fidelity. Today, even a combination of eight wavelengths is insufficient for some users, particularly where the microscope is a shared resource. As shown in figure 3, microscope manufacturers and end users are now starting to use the Galaxy laser combiner as part of a system to generate 10 wavelengths, albeit with three output fibers rather than one. The confocal microscope has certainly come a very long way from early instruments featuring a single 488 nm ion laser!

### Summary

With a history dating back to Galileo, the optical microscope is still a frontline tool in biological research. Today the field is more dynamic than ever, particularly when using lasers. As researchers and instrument designers develop new techniques and different fluorescent tools, laser manufacturers support this effort by creating new lasers with optimized output characteristics for each new scenario.

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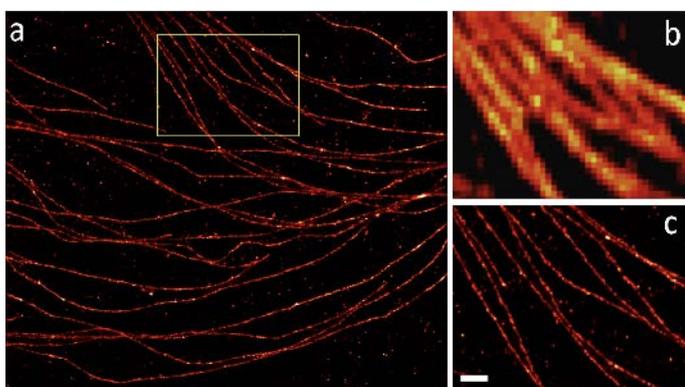


Figure 2: Stochastic microscopy imaging methods such as STORM deliver resolution far beyond the classical diffraction limit. Examples of STORM (a,c) and normal (b) resolution. These images from Professor Zhen-li Huang's research team at Huazhong University of Science and Technology are characterized by 40 nanometer resolution. Scale bar: 500 nm

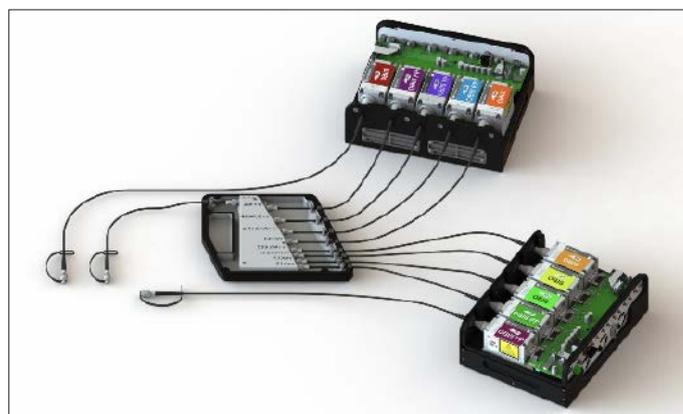


Figure 3: The OBIS Galaxy wavelength combining module allows eight different laser wavelengths to be coupled into one single-mode fiber with plug-and-play connectors. Here, it is shown (with the cover removed) as part of a system to deliver 10 wavelengths in three output fibers