Lasers for Optogenetics and Multimodal Microscopy

Next ultrafast laser generation opens up a diversifying and dynamic range of non-linear imaging applications

Marco Arrigoni

A new type of short-pulse ultrafast laser sources provides extremely wide (680 – 1300 nm) wavelength tunability of the primary output as well as simultaneous high power (1.5 watts) output at 1040 nm. This combination makes lasers such as the Chameleon Discovery ideal for demanding multiphoton excitation applications in optogenetics, multimodal imaging, and other microscopy applications that need two independent wavelengths. In this article we will see why these applications benefit from this performance.

Applications diversity

Neuroscience – particularly optogenetics – and the pursuit of potential diagnostic applications are major drivers behind the development of the latest ultrafast microscopy laser tools recently introduced by leading manufacturers like Coherent. Specifically, these clinical applications now combine two or more non-linear techniques in so-called multimodal imaging, which creates a requirement for wavelength agility and the ability to simultaneously generate pulses at more than one wavelength. Just as important, the increasing use of ultrafast lasers for optogenetics also drives demand for ultrafast lasers with output at more than one wavelength, simultaneously. Most of these applications benefit from long wavelengths and higher available power. Long excitation wavelengths enable deeper imaging where the main depth limit is scatter-induced signal loss; scatter scales as $1/\lambda^4$. High power boosts signal strength enabling deeper images and faster imaging, which is very

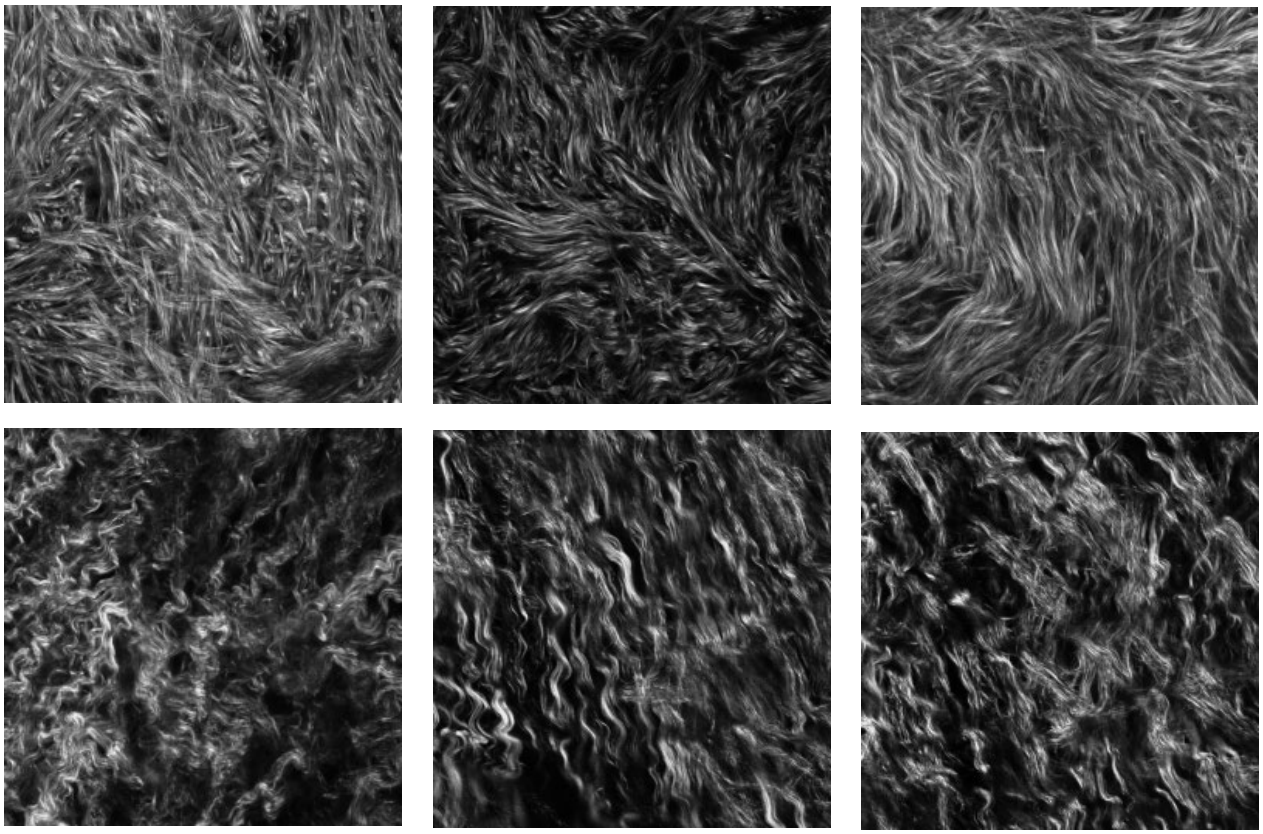

Fig. 1 Normal tissue (above), cancerous tissue (below). These second harmonic generation (SHG) images (single optical sections) were from ex vivo human ovarian tissues. The malignant tumors were high grade serious tumors as classified as pathology. The field size in each case was 170 × 170 microns. The images were acquired at 40 × 0.8 *NA* at zoom 2 using 890 nm excitation. These images were acquired in the forward detection, and isolated with a bandpass filter and detected by a single photon counting photomultiplier. (Source: Campagnola lab, University of Wisconsin-Madison)

important for following biochemical events in real time.

To understand the demands of multimodal imaging, it is useful first to briefly examine some of the commonly used non-linear imaging techniques, often collectively referred to as multiphoton excitation (MPE) that currently rely on femtosecond laser sources and deliver inherent three-dimensional imaging. The oldest and simplest method is two-photon excitation of fluorescent dyes and probes. The last ten years also witnessed an explosion in the use of genetically encoded fluorescent proteins that are transgenically expressed to target specific cellular components. Examples include green fluorescent protein and its enhanced form (GFP and eGFP), yellow fluorescent protein (YFP) and a host of red fluorescent proteins (among which are the mFruit series).

Three-photon excitation is now becoming more popular because it allows to excite at 1 micron or longer wavelengths some of the probes that in two-photon regime would require 700 – 800 nm excitation light. Although three-photon excitation requires more power, the longer wavelengths provide all the benefits discussed above. Importantly, this process can be used to excite most endogeneous fluorophores present in cells (NADH and FAD) and is thus usable for diagnostic purposes.

Two other techniques that enable label-free imaging are second harmonic generation (SHG) and third harmonic generation (THG). Here, the signal is detected as forward or backward scattered light at twice (SHG) or triple (THG) the laser wavelength. SHG occurs whenever a laser with high peak power is focused into non-centrosymmetric structures. Some of these occur naturally, with the most common example being collagen, found in muscle and many other tissues. SHG has become the leading candidate for nascent pre-clinical studies (Fig. 1). Indeed, it probably represents about 80 % of the current research in pre-clinical in-vivo nonlinear imaging.

In theory, THG can occur at most interfaces between different refractive indices, making it useful for imaging cell membranes and other lipid water interfaces. To generate a signal in the visible spectrum, enabling detection by photomultiplier tubes (PMTs) and silicon photodetectors, THG microscopy requires long excitation wavelengths (1.2 – 1.5 µm).

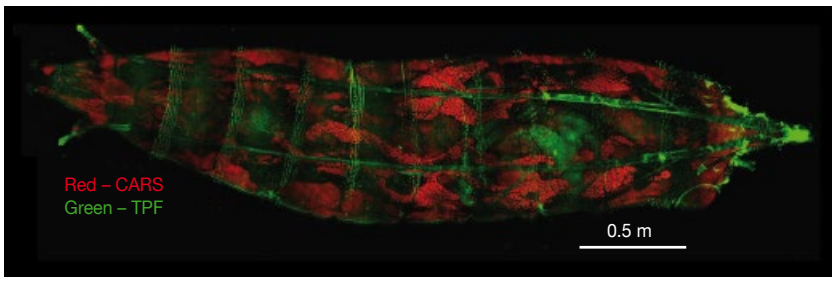

Fig. 2 An example of multi-modal imaging – false color full body image of a drosophila larva. Red is a label-free CARS signal, and green is a two photon fluorescence signal. (Source: Ji-Xin Cheng, Purdue University)

A couple of other label-free techniques are based on Raman scattering and require two different wavelengths simultaneously. These are coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS). CARS and SRS work very well with femtosecond pulses when applied to relatively broadband and isolated molecular vibrations, like the ones associated with lipids, the most common structure imaged with these coherent Raman techniques.

Multimodal imaging

An important trend in non-linear imaging, is the increasing use of multimodal imaging, where two of the above techniques are combined (see Fig. 2). In research applications for example, THG is often combined with two-photon excitation of a fluorescent protein, or two proteins may be imaged in the same sample, e. g., using eGFP and one of the mFruits. One of the other drivers increasing the interest in multimodal imaging is the (eventual) transition to clinical applications. These applications must rely on label-free (e. g., endogeneous fluorescence) imaging, where signals are naturally sparse and weak, compared to fluorescent protein labels. The use of more than one data source in these images will greatly help enhance their otherwise limited information content. As with SRS, multimodal imaging requires two ultrafast wavelengths. And, at least one of these needs wide tunability, as noted before, with the other at a wavelength of broad utility.

Neuroscience and optogenetics

Neuroscience has become the dominant applications space for multiphoton microscopy, with at least half the newly funded applications, because of a focused and increasing government and private funding of this field. Examples include the BRAIN Initiative in the US, the Human Brain Project in the EU, and the Brain/MINDS funding program in Japan.

Within the dynamic field of neuroscience, some of the most exciting work is in optogenetics. Pioneered by Deisseroth's group in Stanford and Boyden's group at MIT, optogenetics is an innovative method where light is used to stimulate specific cells, thanks to a class of proteins – the opsins. When irradiated at the appropriate wavelength, these membrane resident proteins enable ions to flow across the membrane leading to a change in the membrane potential that mimics the normal action potential used for neural signaling. Optogenetics involves using light, often (but not always) in conjunction with microscopy, to activate or silence neural networks in the brain cortex of live animals that have

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been genetically modified to "express" these light-sensitive probes.

An optogenetic experiment on the microscopic scale involves first imaging the neurons, then measuring signaling activity (including timing) in the local neural network following stimulations or silencing of other parts of the network. Signaling is usually measured in term of local Ca^{2+} ion concentration using another type of genetically encoded probes like GCaMP that fluoresce more strongly in the presence of Ca^{2+} ions when activated with a laser beam. The combination of these protocols is usually called all-optical physiology. Ultrafast lasers for multiphoton excitation (MPE) are ideal tools for all three protocols (imaging, stimulation/silencing and detection) because MPE enables deep (1 – 1.5 mm) imaging with full 3D resolution and low photodamage. As a result, larger brain volumes can be studied for extended periods in vivo. And the challenges of separating the stimulation and detection/probing are minimized if different wavelengths are used for these two processes – see Fig. 3.

New ultrafast laser sources

What does all this mean for ultrafast laser sources dedicated to microscopy applications? These trends are creating a demand for

1) Longer wavelengths to enable deeper imaging in live tissue
2) The widest possible tunability to optimally support multiple applications
3) The availability of two separate wavelengths for multimodal imaging and optogenetics-based optical physiology
4) High power to enable faster imaging to follow biological processes in real time

Titanium:sapphire lasers have been the dominant lasers for nonlinear microscopy since its very inception. These compact, tunable sources are still a preferred choice in many applications. To better support multimodal imaging and all-optical physiology studies laser developers have recently looked beyond Titanium:sapphire as a gain material.

One approach is to complement existing Titanium:sapphire lasers by adding a short pulse fixed wavelength oscillator based on ytterbium-doped fiber. An example of this is the Coherent Fidelity launched in early 2014. This laser provides over 2 watts of total power with a pulse-width of < 60 fs at a fixed output wavelength of 1070 nm. Combined with a tunable Titanium:sapphire laser such as the Coherent Chameleon Vision, this set-up provides high power at two separate wavelengths. Specifically, at present a typical multiphoton experiment with individual neuron resolution might involve stimulating then imaging the activity of only a few neurons simultaneously. Ultimately researchers would like more power to simultaneously survey as many as 10,000 neurons which means a column of cortex measuring 250 microns × 250 microns with a depth up to 1 mm. Already, early users of Fidelity are reporting the ability to successfully stimulate several tens of neurons in a single experiment.

New ytterbium-based lasers match applications needs

Within the past year, new ytterbium-based lasers have appeared that incorporate non-linear techniques to generate a widely tunable output. An example is the Coherent Chameleon Discovery with a primary output beam tunable from 680 – 1300 nm. This output is characterized by a pulse-width of 100 fs and delivers up to 1.4 watts, with $M^2 < 1.2$, making it ideal for most multiphoton imaging applications. Furthermore, the Discovery is unique among these next generation lasers in that part of the direct ytterbium power (1.5 watts) is available as a high quality ($M^2 < 1.2$) beam with fixed wavelength at 1040 nm, and short (160 fs) pulse-width, which is also ideal for many multiphoton excitation needs.

In optogenetics, these new lasers offer a near-perfect solution with the ideal combination of output characteristics for these studies; two output wavelengths, short pulse-widths, long wavelength and high power. One approach is to use the fixed 1040 nm output to photoactivate a long wavelength opsin, such as C1V1,which can be very effectively

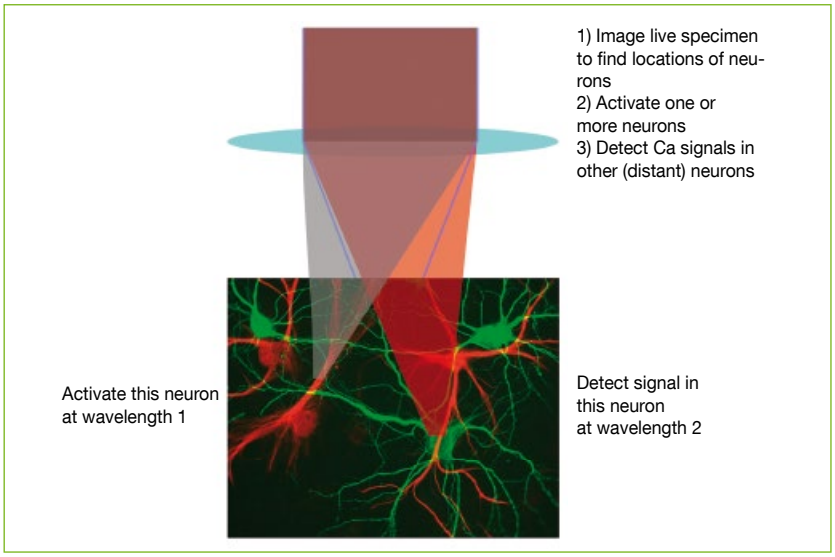

Fig. 3 In a proto-typical optogenetics experiment, two different wavelengths are often used to avoid crosstalk. One wavelength is used to provide photoactivation of target neuron(s) and a second wavelength to detect Ca ion spikes in interconnected neurons. The detection (probe) laser is also used at the very start of the experiment to map the structure of the target tissue before any activation.

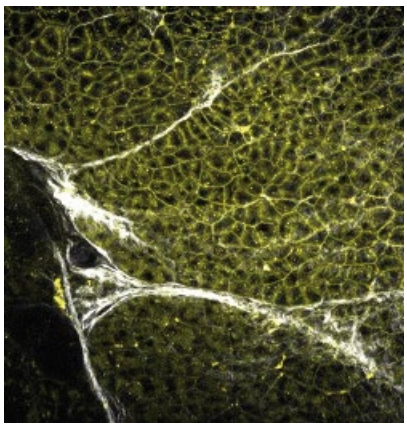

Fig. 4 An example of the benefits of next generation new dual wavelength excitation lasers. Pancreas tissue imaged using SHG at 1040 nm to show collagen surfaces and Raichu-rac biosensor expressed as a FRET probe revealing cell crypts at 830 nm. Image taken with a Coherent Chameleon Discovery laser (Source: Courtesy of Ewan McGhee and Kurt Anderson, Beatson Inst. Glasgow)

activated at this wavelength. (Incidentally 1040 nm can also be used to map the neural structures using a fluorescent protein such as one of the mFruits.) Probing can then be done at 920 nm with the fluorescent calcium indicator family GCaMP, which has low sensitivity above 1000 nm.

An alternative setup reverses the roles of the two laser outputs, where the 1040 nm output probes neurons via a longer wavelength calcium indicator, such as RCaMP, and the tunable output set to around 940 nm to stimulate neurons expressing ChR2 opsin. This approach is expected to become more popular as the RCaMPs, now in their infancy, will be further improved.

These new lasers also match up very well with several combinations of multimodal imaging, both for labeled tissues (see Fig. 4) and for pre-clinical experiments based on endogenous signals only. One example of the latter is to set the tunable output around 750 nm for two-photon fluorescence excitation of the NADH found in every cell, and to use the fixed 1040 nm wavelength to look at collagen for example using SHG. Alternatively, the tunable output can be set to the 1.2 – 1.3 µm region to map hydrophilic/hydrophobic (i. e. aqueous/lipid) interfaces such as membranes using the THG imaging to generate a blue/violet signal in the 400 – 450 nm region. (With shorter excitation wavelengths much of the UV signal would be re-absorbed by the tissue.)

With labeled tissues, a common multimodal use of these new lasers will be to use the fixed 1040 nm output to image one of the mFruit series of red fluorescent proteins. The tunable output can then be set to optimally excited eGFP (enhanced green fluorescent protein) or YFP (yellow fluorescent protein).

Summary

The importance of multiphoton excitation microscopy has long been recognized by laser manufacturers who have supported these applications with a series of Titanium:sapphire based lasers that are packaged and optimized for their unique requirements. Now recent developments in the areas of multimodal imaging and optogenetics have spurred laser manufacturers to look at different gain materials in order to better meet the needs of some of these applications.

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Author

Marco Arrigoni is Director of Marketing (Scientific Market) for Coherent Inc. He joined Coherent in 1988 as an R&D engineer in Santa Clara, CA, and subsequently held positions in international sales, business unit management and strategic marketing. He holds a MSEE from the Politecnico of Milan, Italy.

Marco Arrigoni, Coherent Inc., 5100 Patrick Henry Drive, Santa Clara, CA 95054, USA; E-mail: marco.arrigoni@coherent.com