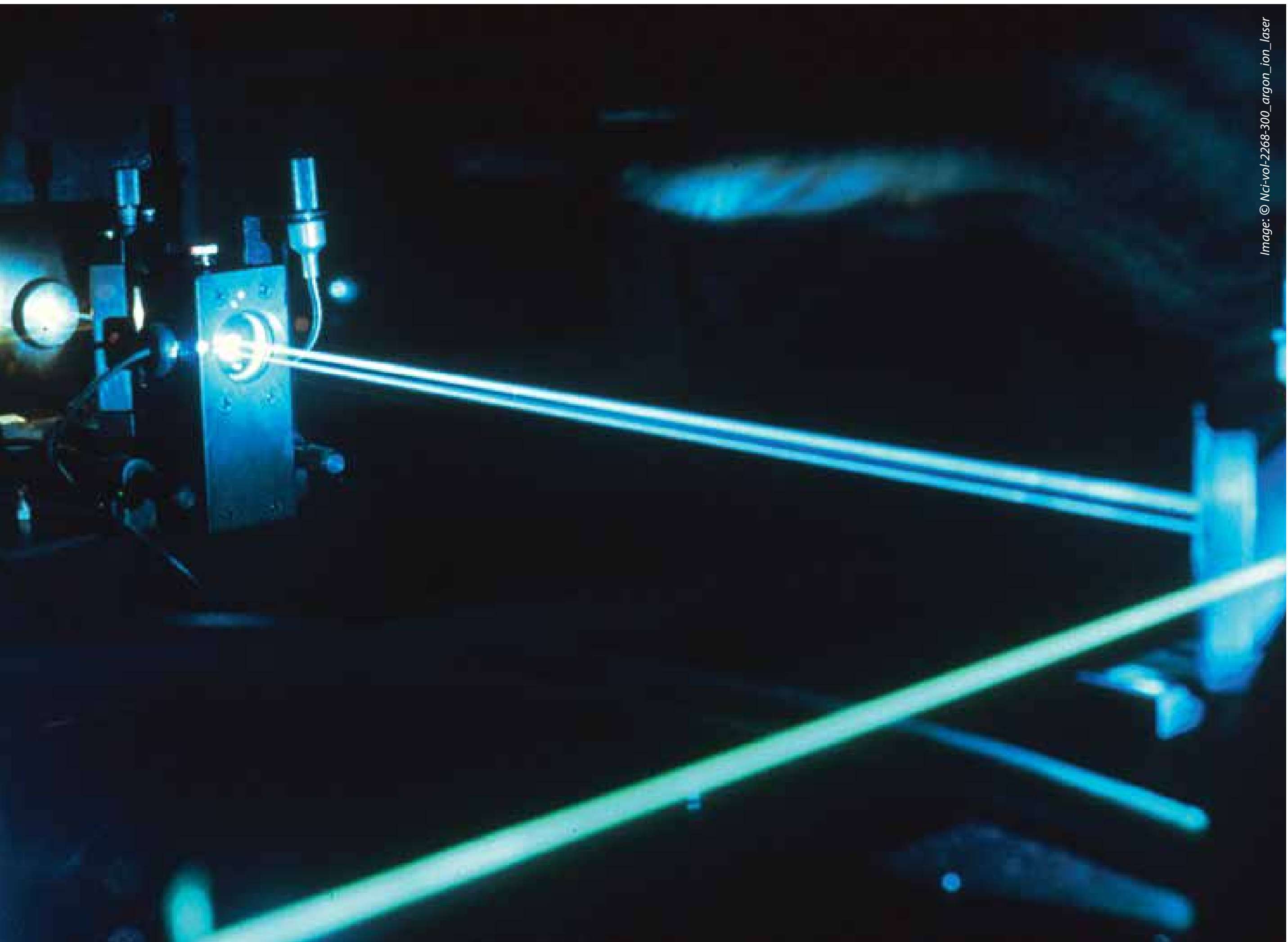

Image: © Nci-vol-2268-300_argon_ion_laser

The Next Step

More and more options to improve imaging capabilities are becoming available to microscopists. But this has demanded a shift in the priorities of laser manufacturers and makers of microscope systems, with the benefits of using ultra-fast lasers quickly emerging

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Although first reported in the early 1990s, two-photon microscopy has, over the last 10 years, become a standard imaging technique in the life sciences laboratory due to the benefits it brings over conventional confocal microscopy. The highly localised excitation of the sample allows for better depth measurement and a reduction in sample exposure to damaging energy while not being imaged. This commoditisation of the technique has, in part, been driven by the reliability and ease of use of the ultra-fast laser delivering short pulses.

The basic design of the microscope system, especially the excitation source, has remained largely unchanged – an ultra-fast laser with a repetition rate of 80-100MHz and a pulse duration in the range of 100-150fs. In recent years, the usability of these lasers has become easier due to less routine need for aligning, cleaning and general maintenance. In the early days of using Ti:Sapphire mode-locked lasers, this daily cleaning and alignment of the cavity meant a delay before microscopy could begin, and the need for a researcher to have photonics as well as microscopy expertise.

Changing Requirements

The needs of the microscopist, however, have moved on, and different options to improve or add to imaging capability demand development from the microscope systems and laser manufacturers. This article will look at one option for

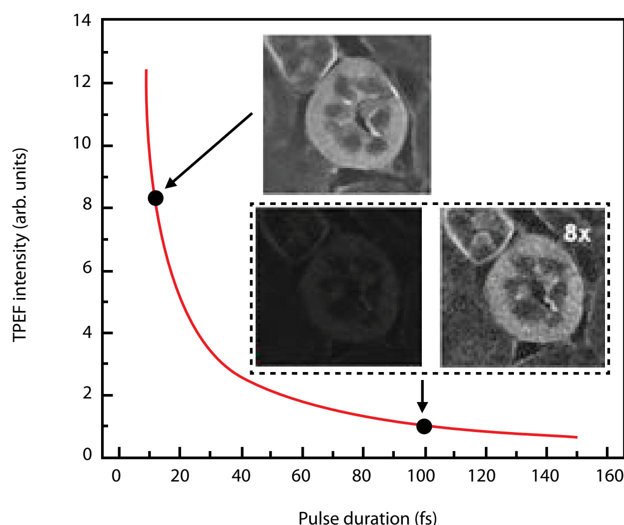

Figure 1: Comparison of 100fs and 12fs pulse duration on fluorescent signal generated on mouse kidney samples. All other parameters including average power remained constant

addressing a number of issues and adding to the imaging capability, namely the use of ultra-short pulses. Few-cycle pulses with durations measured below 10fs have a number of advantages: due to the short temporal duration, they exhibit high peak powers for a given average power and have extremely wide spectral bandwidths. This allows for enhanced excitation efficiency, reduction in photo damage and access to a wide range of fluorophores without the need for wavelength tuning or increased average power (1). There is also evidence to demonstrate the ability of the broad spectral output to promote auto-fluorescence in unlabelled samples – a benefit of particular interest to researchers looking at living cells that require the most natural performance from their samples (2).

In two-photon microscopy applications, the energy needed to achieve fluorescent excitement of a dye comes from the simultaneous arrival of two photons carrying half the necessary energy. With simultaneous meaning within ~10^{-18}s, the probability of a fluorescent event occurring is far lower than in a conventional single photon system. In order to maintain the fluorescent signal intensity and, hence, image quality, the photon density at the focus needs to be far higher than in a single photon or a confocal setup. The probability of a fluorescent emission event increases quadratically with excitation intensity, which corresponds to a necessary photon flux increase in the order of 10^6. This only occurs at the focal point of the ultra-fast laser beam, and by extension the higher the photon flux, the higher the probability of an excitation event occurring. This is true up to the point of fluorophore saturation. In an early paper, Tang S *et al* reported an inversely proportional relationship between fluorescent signal and pulse duration, resulting in an increase of ~160% in imaging depth available from a <20fs compared to a 120fs pulse (3). Additionally, Xi P *et al* reported an eight-fold increase in signal generation when imaging a mouse kidney sample between 100fs and 12fs (see Figure 1) (4).

So why do standard two-photon excited fluorescence lasers remain at ~100-150fs? Ultra-short, few-cycle pulse durations of <10fs have been available for a number of years, and their peak power is far higher than for an equivalent long pulse laser, allowing deeper imaging and lower photo damage. It is the long-term energy dispersion of the average power that causes the photo damage effects, rather than high-intensity peaks over relatively short time periods.

Overcoming the Obstacles

The challenge of the short pulse laser is preserving the duration of the pulse through the optical set-up of the microscope, where highly dispersive objectives and other optical elements will broaden the pulse in the time domain. By definition, ultra-short laser pulses have very wide, often octave-spanning spectral bandwidths, so ensuring the <10fs pulse available from the laser and at the sample plane (focus of the objective) can be a complex business. This dispersion of the wavelengths – known as group velocity dispersion (GVD) – elongates the pulse in the time domain, therefore reducing the peak power and, hence, the excitation efficiency at the sample. In contrast, longer pulses – such as 100-150fs – have a far narrower range of contributing wavelengths, meaning less dispersive broadening through the necessary optics. However, these pulses are intrinsically limited, since they cannot be compressed further to gain the benefits associated with the shorter pulses.

The most common method of dealing with this group velocity dispersion is to reverse the effects of all the different media the pulses will encounter before they start the journey through them. This is termed negative GVD, or pre-chirping. It is a rule of unintended consequences that in order to create the tight focal point of the ultra-fast beam, a high numerical aperture lens and many optics are required – all made from highly dispersive glass, which can lead to group delay dispersion (GDD) values in the order of 5,000-10,000fs^2. This exacerbates the problem and increases the demands on any pre-chirping set-up. It is this additional experimental design that has perhaps held the short pulses back in laboratories where the focus is on microscopy, rather than photonics. Also, using ultra-short pulses of <10fs duration, the dispersion of air though minor also has to be compensated for, and a change of the beam propagation distance in air would need a tweak in the pre-chirping.

What Next?

A photonics lab would generally model the different optical elements in the set-up, derive a theoretical GDD and therefore the pre-chirp needed using software (5), then install the recommended dispersion compensated mirror and wedge (6) combinations needed to achieve the calculated result. If the maximum efficiency and performance is required, the lab may also install a pulse characterisation device such as a SPIDER (7) that offers real time pulse characteristics, enabling fine tuning of the dispersion compensation to achieve the shortest temporal pulse possible – but these can be cumbersome to use in a microscope set-up and need aligning to the beam.

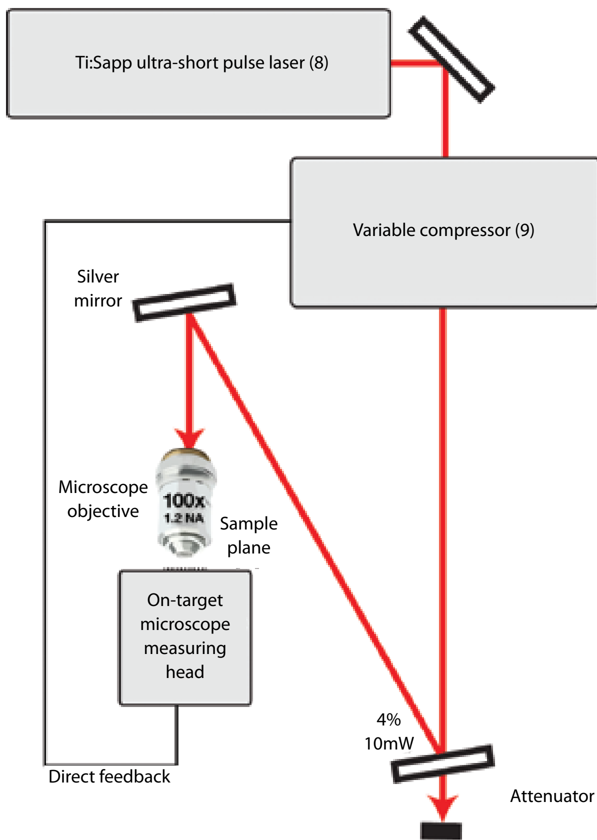

Figure 2a: Experimental set-up, including a venteon ultra laser oscillator, a d-scan system and a pair of objectives

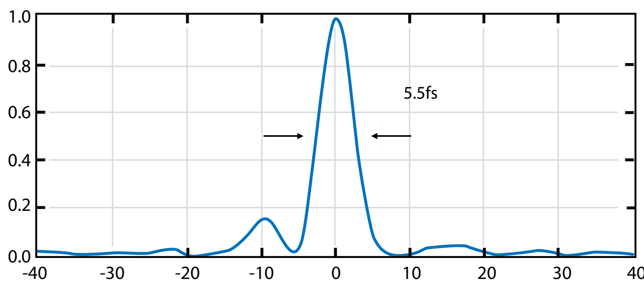

Figure 2b: Retrieved temporal profile from 63x objective. Pulse duration is 5.5 ± 0.1fs full width at half maximum

In recent work, the ability to use the venteon ultra (8) short pulse laser and the d-scan (9) pulse characterisation and compression unit has been reported (10). The venteon ultra brings the capability of <5fs transform-limited pulses with actual measured pulses of <5.5fs, thus offering the benefits of enhanced excitation efficiency and direct excitation of multiple fluorophores due to the broad spectral output, as mentioned earlier in this article (3). Here, the authors describe the use of the venteon ultra and the d-scan to compensate for the dispersion caused by a standard 63x, 0.75 numerical aperture (NA) objective. Being smaller, and having a direct feedback from the 'point of use' characterisation

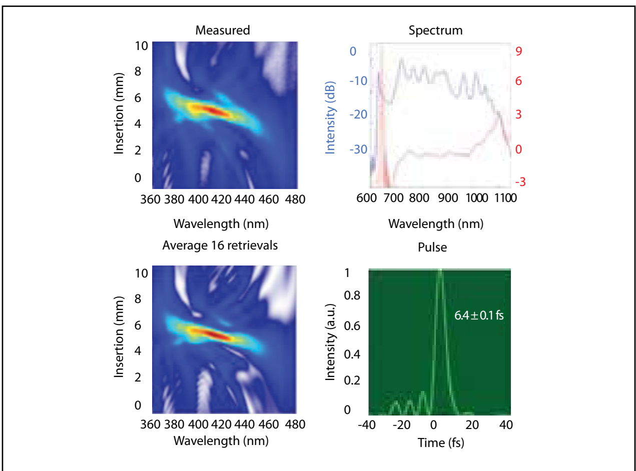

Figure 3: Measured (top left) and retrieved (bottom left) d-scan traces. Top right: measured spectrum (blue curve) and retrieved spectral phase (red curve). Bottom right: retrieved temporal profile. Pulse duration is 6.4 ± 0.1fs full width at half maximum

unit to the compensation module, the d-scan was able to achieve a pulse after the objective of <5.5fs. Figures 2a and 2b show the set-up and result.

They further reported on the compensation available from a 100x, oil-immersed objective (NA of 1.15), achieving a post-lens pulse duration of 6.4fs at the focus of the objective (see Figure 3).

Moving Forward

By using modern ultra-fast lasers, the difficulties once associated with their care and maintenance has largely been eliminated by design stability, production process improvements and the general advancement of the technology. Ultra-short pulses, as available from lasers such as the venteon ultra, can offer significant benefits to the two-photon microscopist. Deeper images can be obtained and higher contrast can be achieved due to the higher photon flux intensity experienced at the focus of the beam.

A wide range of fluorophores are accessible without the need for wavelength tuning due to the octave spanning (>400nm) spectral bandwidth; multiple fluorophores can be excited in one imSage; auto-fluorescence becomes useful; and all without the need to increase the average power and the consequent increase in photo damage and decreased imaging times. The more complex problem of pulse duration maintenance and compensating for the dispersive effects of the microscope optics can be solved by a number of alternative methods – from a low-cost software/mirror/wedge combination to semi-automatic pre-chirper modules, or fully automatic characterisation and feedback units such as the d-scan.

The benefits of ultra-short pulses are now available to the microscopy lab and the microscopist without the need for photonics expertise, resulting in huge advances to the life sciences industry as a whole.

Image: © Quantum_dots_630_nm_pumped_with_green_laser_02

Acknowledgement
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Andrew Wells graduated from De Montfort University, Leicester, with a BSc Honours degree before moving into the commercial environment. Having worked in a number of large companies within the photonics and scientific industry, Andrew joined Laser Quantum in 2011 as the Sales and Marketing Manager. He is responsible for sales in many parts of the world and ensuring the distribution partners have received advanced training to support customers. Andrew also manages the marketing team, who are in charge of delivering interesting and relevant information to their global customer base.
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