Whitepaper

Benefits of ultrashort pulses in two-photon microscopy

***Abstract**: Two-photon microscopy has enabled the imaging of live tissue and cells to higher depth than conventional methods whilst minimising the risk of photo-bleaching and sample damage. Femtosecond lasers with ultrashort pulse and ultrabroad spectrum, such as Laser Quantum's **venteon ultra**, can be easily coupled to a compression and characterisation module to exploit all the advantages they can offer, such as enhanced excitation efficiency, direct access to a wide range of fluorophores or ultrahigh time resolution.*

Introduction

Multiphoton microscopy, also known as two-photon microscopy, is an imaging technique invented by Denk, Webb and co-workers (Denk et al, 1990) relying on the simultaneous absorption of two photons from a laser beam spatially focussed into the sample plane to emit a fluorescence signal. The laser beam must deliver high peak power and short pulse duration in order to deliver brighter images and a high flux of photons, thus increasing the probability of two photons being absorbed simultaneously at the same location (Pestov et al, 2010). This can be achieved by using a mode-locked femtosecond laser, which produces high pulse peak power at an average power low enough to minimise damage to the sample. Due to their broad spectrum, ultrashort laser pulses allow the simultaneous excitation of the most common fluorophores, thus removing the need for wavelength tuning (Pestov et al, 2010).

Laser Quantum's **venteon ultra** exceeds all those requirements by delivering greater than 240 mW of sub 6-fs short pulses with an unrivalled bandwidth greater than 400 nm (FWHM). As the probability for two-photon excitation is inversely proportional to the pulse duration, few-cycle pulses result in enhanced efficiency of nonlinear processes – thus reducing detrimental effects on living cells.

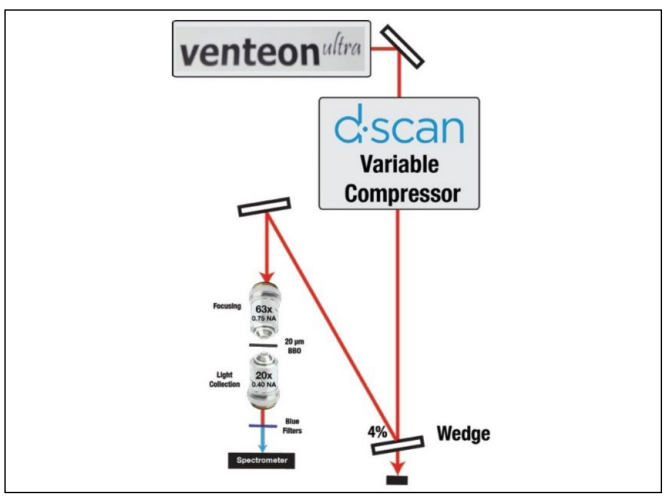

Figure 1a: Experimental setup, including a venteon ultra laser oscillator, a d-scan system and a pair of objectives.

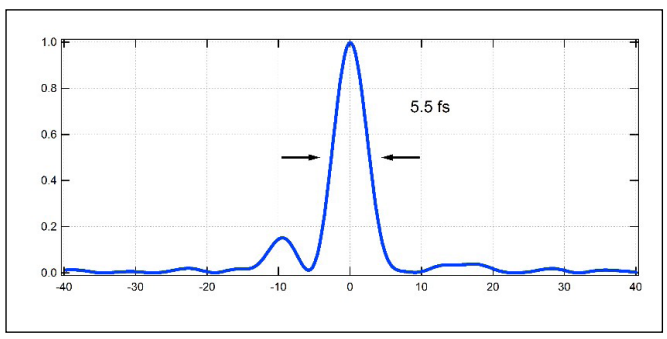

Figure 1b: Retrieved temporal profile from 63x objective. Pulse duration is 5.5 ± 0.1 fs (FWHM).

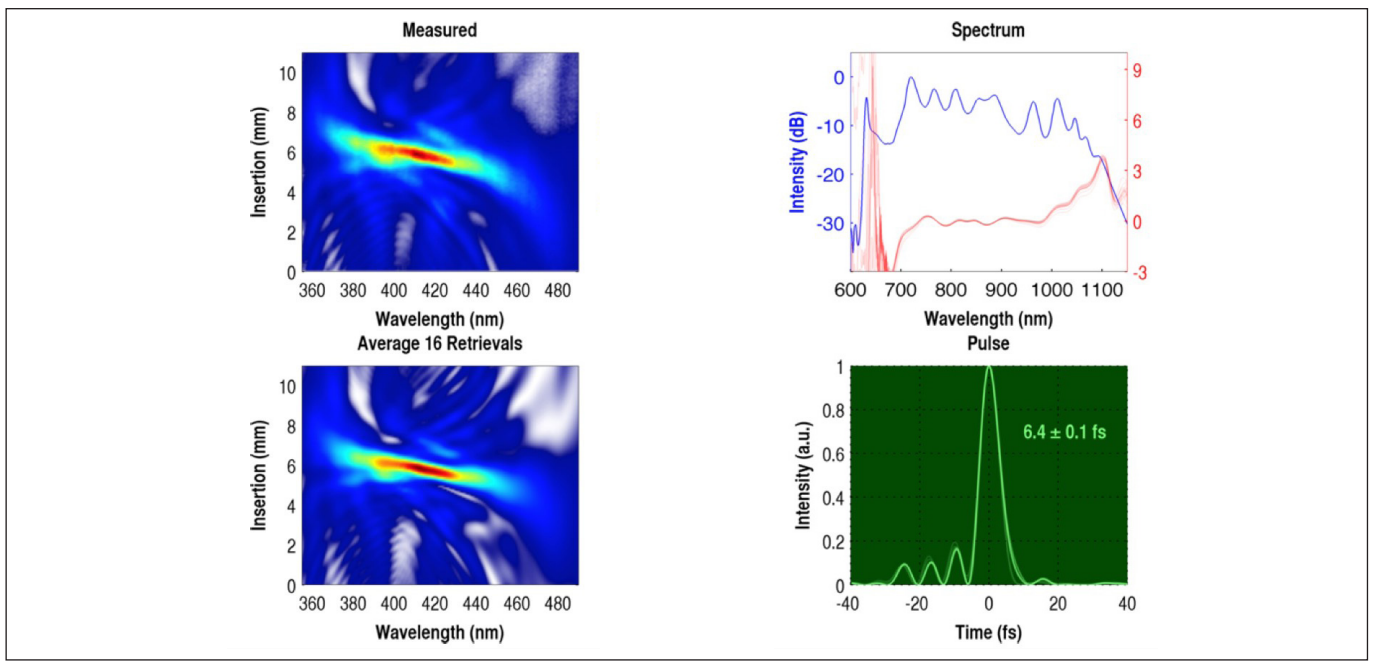

Figure 2: Measured (top left) and retrieved (bottom left) d-scan traces. (Top right) measured spectrum (blue curve) and retrieved spectral phase (red curve). (Bottom right) retrieved temporal profile. Pulse duration is 6.4 ± 0.1 fs (FWHM).

Yet, there is one major obstacle to deal with when using ultrashort pulses in two-photon microscopy. Ultrashort pulses suffer from undesirable temporal stretching of the pulse introduced by the set of optics composing the microscope, known as chirp due to positive group velocity dispersion (GVD). This means ultrashort pulses must be pre-chirped with negative GVD to compensate for the stretching and hence obtain Fourier-limited pulses (thus with high peak power required for the nonlinear processes behind multiphoton microscopy) at the focus of the sample. By using dispersion compensating mirrors such as Laser Quantum's **venteon DCM** mirrors pair, the desired amount of pre-chirp is achieved by bouncing off the output beam multiple times.

Measuring and pre-chirping ultrashort pulses in real time on the focus of a microscope objective can prove challenging. Sphere Ultrafast Photonics' d-scan is an inline, compact and easy to use device allowing simultaneous measurement and pre-chirping of ultrashort pulses (via its integrated **venteon DCM** mirrors pair) so end-users can optimise the pulse shape for different microscope configurations.

Experimental setup and results

Two-photon microscopy technology uses microscope objectives with extremely large positive group velocity dispersion (GVD), of the order of thousands of fs2. Dispersion compensation of these objectives requires introducing a correspondingly large negative pre-chirp. This is very challenging, but can be achieved with the **venteon DCM** mirrors pair.

With an easy setup combining a **venteon ultra** and a custom d-scan (see Figure 1a), it is possible to achieve a very well compressed 5.5-fs pulse duration after a standard 63x, 0.75 NA objective (see Figure 1b).

The d-scan is not only able to measure the pulse duration at the sample, but can directly retrieve the spectral phase and thus gives vital information to analyse the suitability of the compressor. This is especially important for compensation of high NA objectives introducing a GVD greater than 3000 fs2 which has to be compensated up to the fourth order to achieve sub-7 fs pulses in the sample plane. An example of the applicability of the **venteon ultra** and d-scan combination is depicted in Figure 2 for the case of a 100x, oil immersed objective (NA of 1.15). This 6.4-fs pulse duration is a major achievement for such a high NA objective with around 3600 fs2 of GVD and the d-scan reveals that the longer pulse duration in comparison to the 63x objective is mainly caused by a slight mismatch of third order dispersion.

Conclusion

Ultrafast lasers are widely used in many applications and an important example is the technique of two-photon microscopy. It produces high quality images, in particular in biomedical applications where it is important to image deeply into the tissue, in vivo and with high sensitivity. The key to achieve the best quality is to use the shortest possible pulses (below 7 fs). These ultrafast pulses are very demanding and need to be controlled to assure they have the right properties before they are used to image, and very importantly, without damaging, any tissue with the maximum possible resolution. The combination of Laser Quantum's **venteon ultra** laser and Sphere Ultrafast Photonics' d-scan technology opens a new era for such applications as biological probing and imaging as well as high-resolution two-photon microscopy and Coherent anti-Stokes Raman scattering spectroscopy (CARS). It has been demonstrated that reducing the temporal profile of the laser pulses increases resolution and gives more contrast to the images taken with this technology (Dantus et al, 2009). In particular, two-photon microscopy will benefit from this system for studying neurological degenerative diseases allowing for real time imaging of the brain with unprecedented resolutions.

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References:

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