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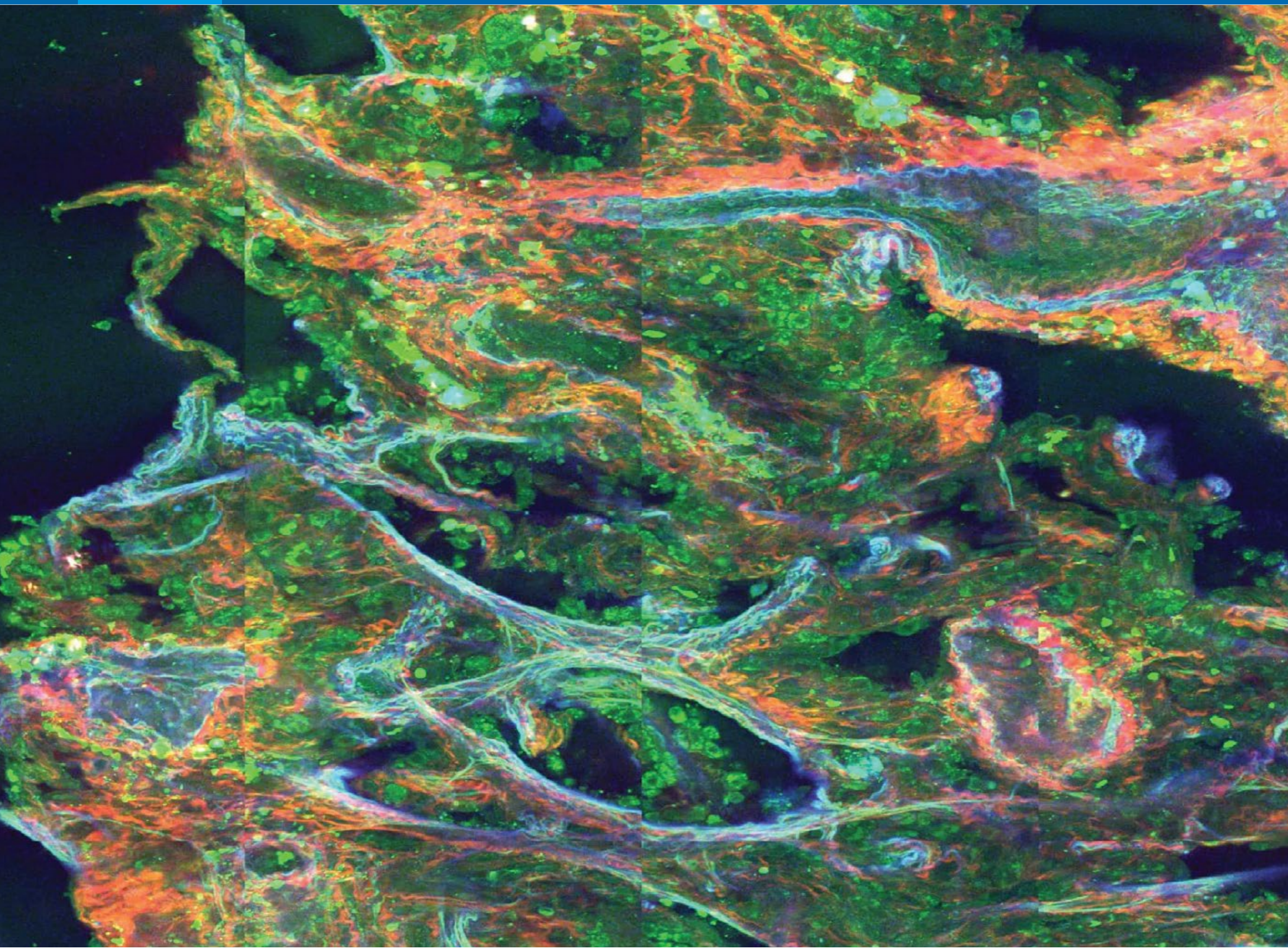


Revealing cellular mysteries with ultrafast lasers

Ultrafast lasers enable rapid cancer diagnosis with simultaneous two and three-photon imaging

A new generation of compact ultrafast laser systems capable of simultaneous two and three-photon microscopy – including two-photon fluorescence (2PF), second harmonic generation (SHG), three-photon fluorescence (3PF), and third harmonic generation (THG) imaging – offer exciting potential to accelerate biomedical research and clinical diagnostics.

About the authors

Oliver Prochnow is CEO of Valo Innovations, a part of Hübner Photonics. He has more than 15 years of experience in the field of ultrafast fiber lasers and amplifiers including a PhD from Laser Zentrum Hannover from the Ultrafast Photonics Group. He is author or coauthor of more than 45 scientific publications.

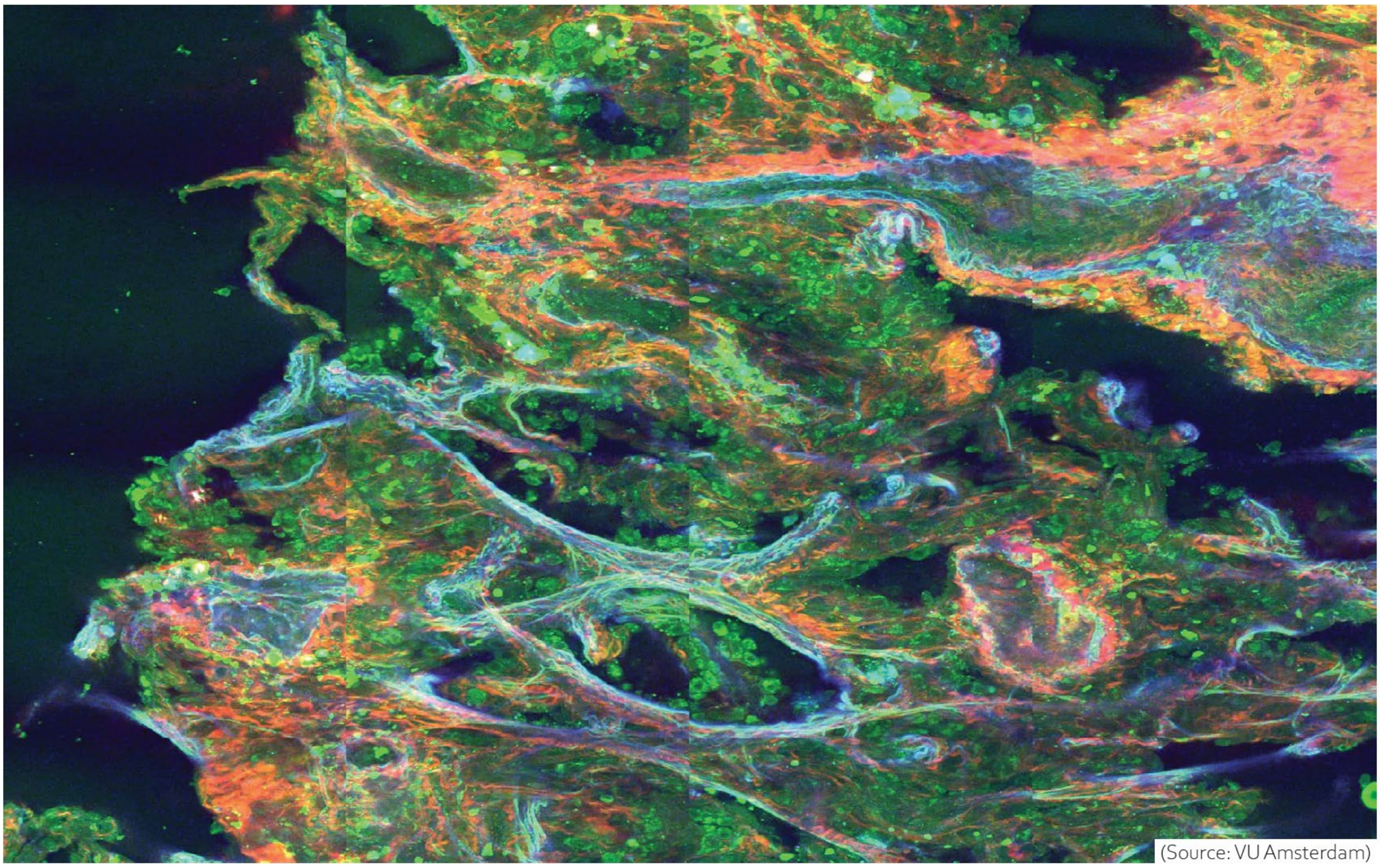
Laura van Huizen is an application development manager at Flash Pathology. During her doctoral studies at the Vrije Universiteit Amsterdam, she was the first to introduce a portable higher harmonic generation microscope into the hospital, validating its use for various biological tissues and diseases. After a postdoctoral position at Aarhus University, she returned to Amsterdam to join the Flash Pathology team

3D in-depth visualization

Femtosecond lasers are a critical enabler of higher-order harmonic generation (HHG) imaging, commonly referred to as multiphoton microscopy. Unlike conventional single-photon excitation, multiphoton processes require two or more photons to arrive at the exact same location and time to simultaneously excite a molecule – a highly improbable event under normal conditions. This nonlinear interaction becomes feasible only with the extremely high peak intensities of ultrashort laser pulses.

As a result, femtosecond lasers make it possible to achieve precise, localized excitation within biological tissues, with minimal photodamage and improved imaging depth – hallmarks that make multiphoton microscopy uniquely powerful in biomedical imaging. Since the interaction occurs only at the focal point, the excitation volume can be scanned throughout the sample, enabling intrinsic three-dimensional imaging without the need for confocal pinholes or outof-focus light rejection.

Pulse duration is the key factor to improving imaging quality. Reducing the pulse duration by a factor of five while maintaining constant average power results in roughly a five fold increase in signal for two-photon absorption. Three-photon excitation is even more dependent on peak intensity, scaling with the cube of the intensity and inversely with the square of the pulse duration. Consequently, the same reduction in pulse duration can produce up to 25 times higher signal for three-photon processes. What is more, if peak power is held constant, shorter pulse durations allow for a proportional reduction in average power compared to systems with longer pulses. This reduction in average power significantly limits sample heating and photobleaching, enabling long-term imaging of live cells with minimal damage – an essential benefit for sensitive biological applications. In general, shorter pulse durations are accompanied by broadband optical spectra. To achieve pulse durations as short as thirty femtoseconds, the spectrum typically spans the range of approximately 990 – 1,140 nm.



(Source: VU Amsterdam)

Figure 1:

In this lung tissue image, third harmonic generation (green) visualizes cells and other structures, second harmonic generation (red) visualizes collagen fibers, while two-photon excitation (blue) visualizes autofluorescence, mainly from elastin fibers (image width: 800 µm)

This broad spectral range introduces a previously unattainable capability: the portion of the spectrum above 1,080 nm can efficiently generate three-photon signals that are transmitted through standard microscope objectives. In contrast, three photon signals generated from wavelengths below 1,080 nm are blocked due to UV absorption in standard objective lens material, limiting their transmission (Fig.2). This makes the use of broader spectra not only beneficial for pulse compression but also essential for unlocking advanced three-photon imaging through conventional optics.

The VALO Femtosecond Series represents a new class of ultrafast fiber lasers, delivering exceptionally short pulse durations and an ultrabroadband optical spectrum, all in a compact easy-to-use form factor, making them suitable for simultaneous two and three-photon excitation.

Thanks to a distinctive design, these lasers produce temporally clean sub-40 femtosecond pulses (Fig.3) with an exceptionally broad spectrum (Fig.4). Compared to conventional systems, their shorter pulse durations yield significantly higher peak power resulting in unmatched imaging contrast.

Engineered for reliability and ease of integration, these robust fiber lasers feature a built-in dispersion precompensation unit, ensuring the shortest pulses reach the sample after passing through a microscope objective. They also operate without the need for water cooling, simplifying the setup and operation.

Fast and accurate cancer diagnosis with higher - harmonic generation imaging

Higher-order harmonic generation (HHG) microscopy systems can ultimatelybe used in many biomedical research or clinical diagnosis applications as they provide high-resolution and high-contrast imaging capabilities. This technique is a nonlinear optical microscopic technique that enables label-free imaging of fresh unprocessed biological specimens.

Imaging biological samples with traditional microscopy requires sample slicing and staining to create contrast in the tissue. The nonlinear mechanisms generated by femtosecond laser pulses, however, eliminate the need for labeling or sample preparation, revealing molecular and structural details within tissue and cells while leaving the sample intact.

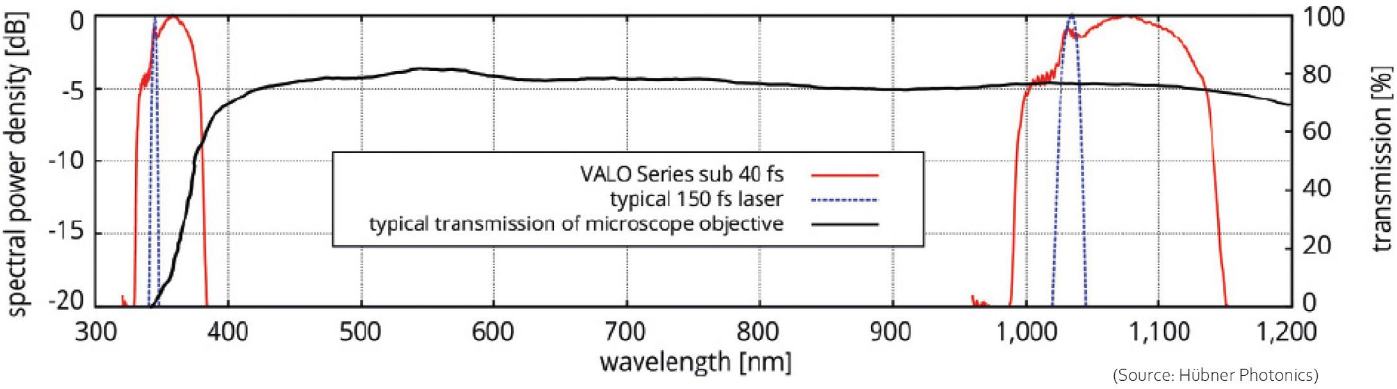


Figure 2:

Fundamental and third-harmonic generation (THG) spectra of a 30 fs broadband fiber laser (red) compared with standard 150 fs lasers. The solid black line shows the typical transmission characteristics of a standard microscopy objective. Only a THG spectrum generated from wavelengths of above 1,080 nm will be transmitted.

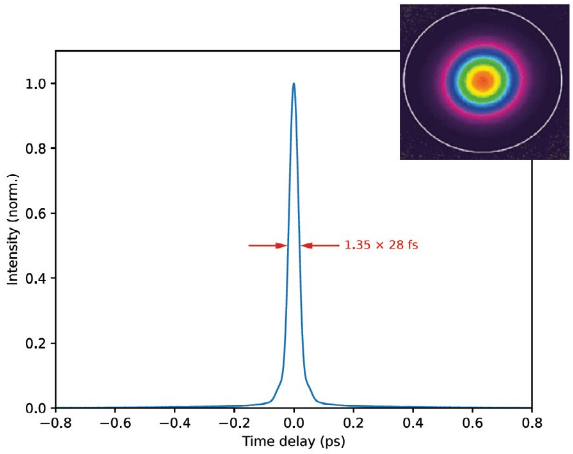


Figure 3:

Typical temporal pulse profile of a 28 fs pulse with very low pulse pedestal. The inset shows the typical beamprofile. (Source: HÜBNER Photonics)

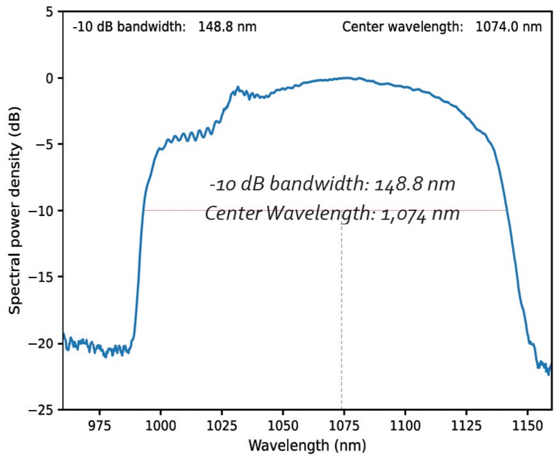


Figure 4:

Typical optical spectrum of Hübner Photonics' VALO Femtosecond Series lasers. (Source: HÜBNER Photonics)

One key application is cancer diagnosis, for example during bronchoscopy procedures; it can be challenging to obtain adequate material for diagnosis. The standard histopathological analysis can take several days to provide diagnostic results and, in cases where no diagnosis can be made, the patient might have to come back for a repeated procedure. This indicates the clinical need for rapid intraoperative feedback on biopsies.

Higher-order harmonic generation imaging, on the other hand, can rapidly visualize unprocessed tissue samples, enabling diagnosis in situ. A recent study demonstrated that it could image a biopsy sample and provide feedback just six minutes after excision, with an accuracy of 87 % – enabling immediate decisions as to whether a further biopsy is required [1].

The typical HHG microscopy system's impressive diagnostic performance is partly due to its ability to generate four nonlinear signals simultaneously using a single ultrafast femtosecond laser: second and third-harmonic generation plus two and three-photon fluorescence. The system then uses filters to spectrally separate these signals, which provide complementary diagnostic information. SHG, a coherent nonlinear process, arises in noncentrosymmetric structures such as collagen fibers, microtubules, and other ordered biological assemblies. This makes it particularly useful for imaging connective tissues, cytoskeletal structures, and fibrillar proteins with high contrast and specificity.

On the other hand, THG occurs at interfaces with refractive index mismatches or in regions of material inhomogeneity, providing detailed imaging of cell membranes, lipid-rich structures, and organelle boundaries. Two and three-photon fluorescent signals are generated by autofluorescent molecules, such as FAD and NADH, visualizing elastin and cell cytoplasm.

Towards future clinical applications

Once fully realized in the clinic, a higher-order harmonic generation imaging system will provide an invaluable tool for rapid in situ tissue analysis during bronchoscopy procedures or other procedures where tissue is excised. The unique combination of four nonlinear imaging modalities, made possible with a single compact femtosecond laser, delivers complementary diagnostic information and is poised to revolutionize rapid medical diagnostics.

References

L.M.G. van Huizen, et al.: Rapid on-site histology of lung and pleural biopsies using higher harmonic generation microscopy and artificial intelligence analysis. Modern Pathology (2025).

About the authors

Hübner Photonics is an international organization with offices in Sweden, Germany and the US. The company supplies high performance lasers to leading instrumentation manufacturers, innovative start-ups, universities, and research labs around the world. Fiber laser manufacturer Valo Innovations became a part of Hübner Photonics in 2021. www.hubner-photonics.com

Flash Pathology is a Netherlands-based start-up, originally spun out of the Vrije University. The company is developing a compact, affordable higher harmonic generation microscope that will enable real-time visualization of unprocessed tissue in a clinical setting. www.flashpathology.com