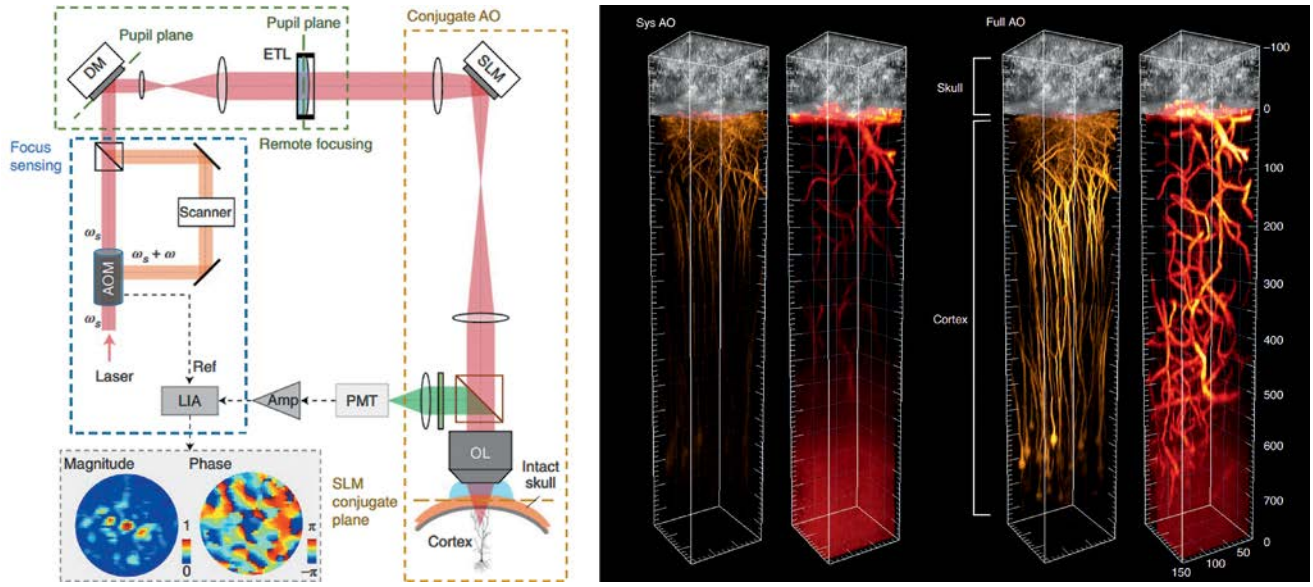
Examples of Nonlinear Microscopy

FUNCTIONAL 3P NEUROIMAGING

Recording of real-time single-neuron activity in the deep brain layers of awake animals is crucial for understanding behavior as well as brain connectivity and function. These applications have been advanced by neuron imaging and stimulation using high-power, high-pulse-energy, medium-repetition-rate lasers tunable in the SWIR range, which spans the biological transparency windows at 1.3 µm and 1.7 µm. For two- and three-photon-excited fluorescence (2PEF, 3PEF) and harmonic-generation (SHG, THG) imaging in deep tissues, dispersion-controlled femtosecond pulses from ORPHEUS line OPAs and microscopy-dedicated CRONUS-2P and CRONUS-3P lasers are truly a state-of-the-art choice.

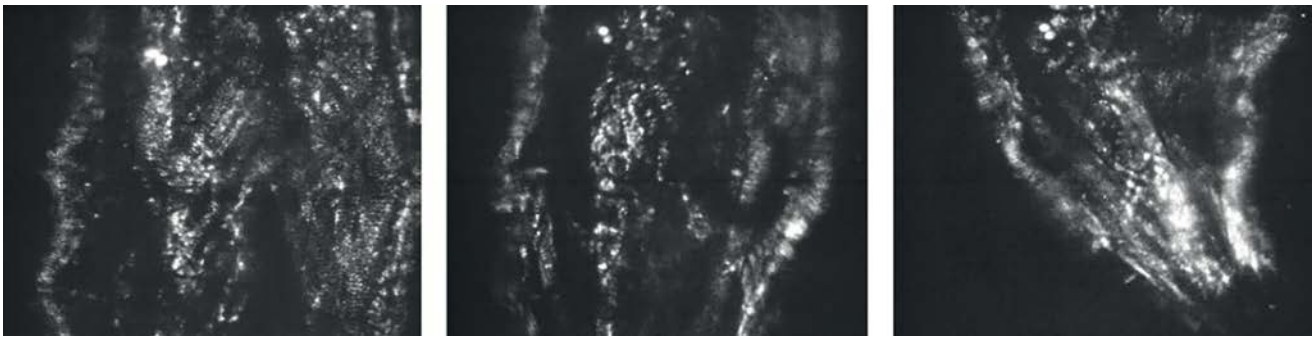


3P microscopy with adaptive optics for focus sensing and shaping to compensate for both aberrations and scattering. ORPHEUS-F excitation at 1300 nm enabled imaging up to 1.1 mm below the pia within the intact brain.

Courtesy of Jianan Y. Qu group, the Hong Kong University of Science and Technology. Source: Zh. Qin et al., Deep tissue multi-photon imaging using adaptive optics with direct focus sensing and shaping, Nature Biotechnology 40 (2022).

LABEL-FREE *IN VIVO* WIDEFIELD SHG IMAGING

Nonlinear excitation requires very high peak intensities and thus has been traditionally limited to laser-scanning microscopy using tightly focused beams. For some *in vivo* and high-throughput applications, however, laser scanning is too slow. With improved femtosecond laser technology delivering ever-increasing average power, it is now possible to excite nonlinear signals over a large area using widefield nonlinear microscopy. Since optimal excitation conditions are application dependent, the tunable repetition rate and pulse energy of the PHAROS and CARBIDE lasers as well their industrial reliability and low-noise performance are key parameters when building widefield setups.

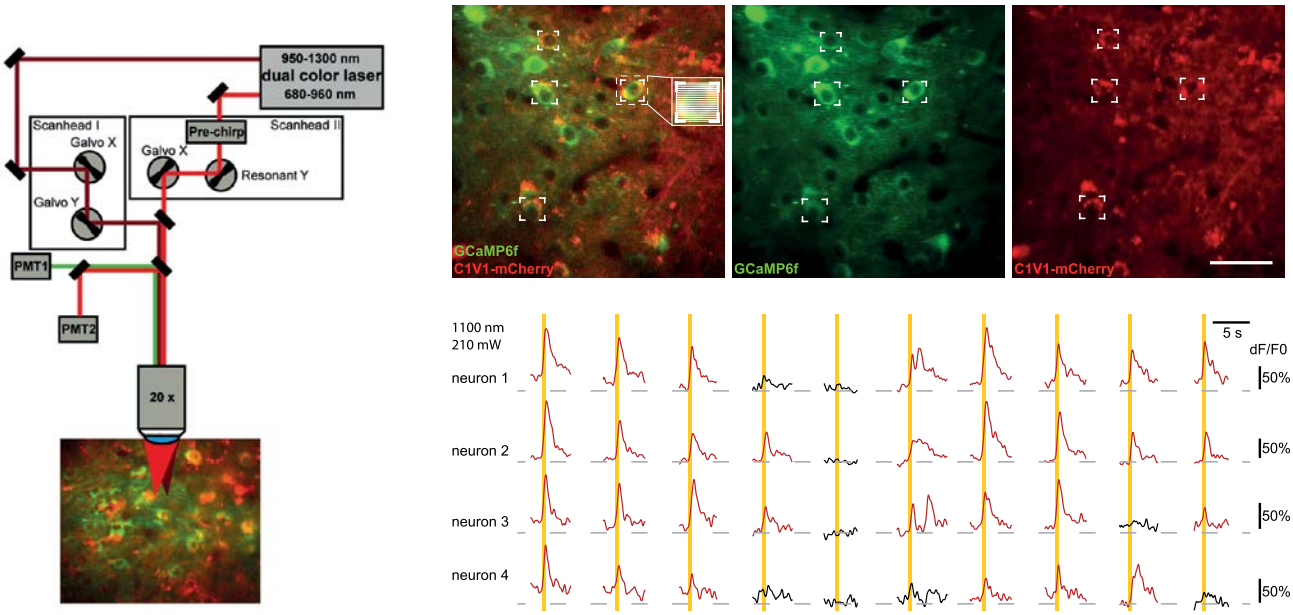


Label-free *in vivo* widefield SHG imaging of fruit fly larva using PHAROS femtosecond laser.

Courtesy of Virgis Barzda group, University of Toronto.

2P OPTOGENETICS

Despite the advances in 3-photon excitation sources providing longer wavelengths and higher pulse energies, certain imaging challenges are still better addressed by tunable high-repetition-rate oscillator-based lasers. This is especially true when imaging speed is the primary factor. For these applications, the CRONUS-2P laser offers the ultimate solution with its optically synchronized three-outputs, two of which are independently tunable. A three-beam source enables a variety of multiphoton excitation pathways, many of which are inaccessible using traditional single- and two-beam solutions. Furthermore, independent tunability of the two beams enables new coherent Raman scattering modalities.

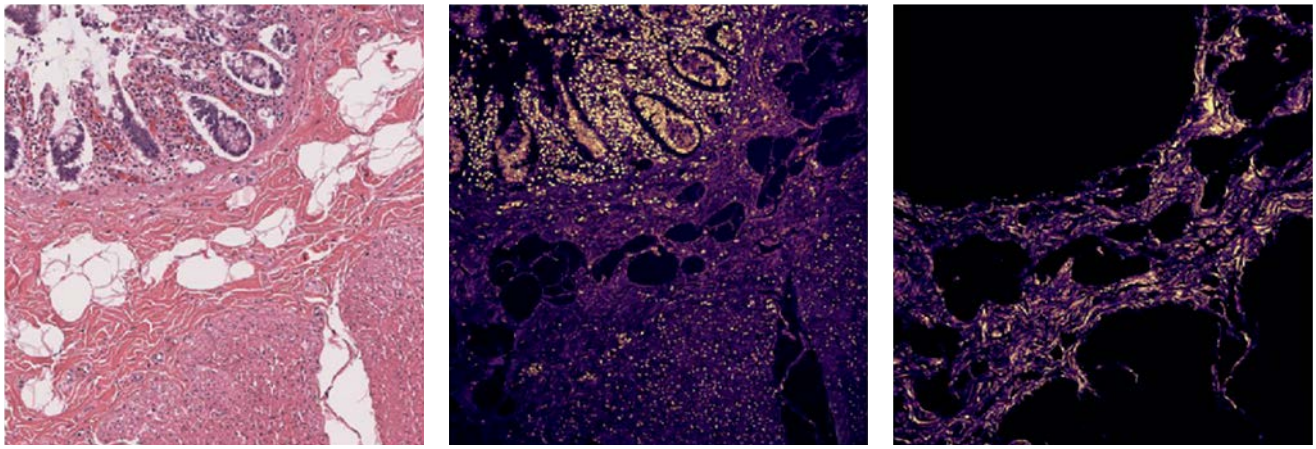


2P optogenetic stimulation of individual neurons using CRONUS-2P.

Courtesy of Albert Stroh group, University Medical Center Mainz and Leibniz Institute for Resilience Research. Source: T. Fu *et al.*, Exploring two-photon optogenetics beyond 1100 nm for specific and effective all-optical physiology, iScience 24 (2021).

RASTER-SCANNING 2P/3P MICROSCOPY

For applications requiring a fixed-wavelength femtosecond laser, such as multiphoton-driven fluorescence (MPEF), excited at 1 µm, and harmonic-generation (SHG, THG) microscopy, the FLINT oscillator is a high-performance solid-state source in a proven, industrial-grade package and a compact footprint. In particular, the FLINT oscillator provides stable 24/7 operation with excellent noise performance, characterized by a RIN that is <140 dBc/Hz above 200 kHz and shot-noise-limited at -160 dBc/Hz above 1 MHz.

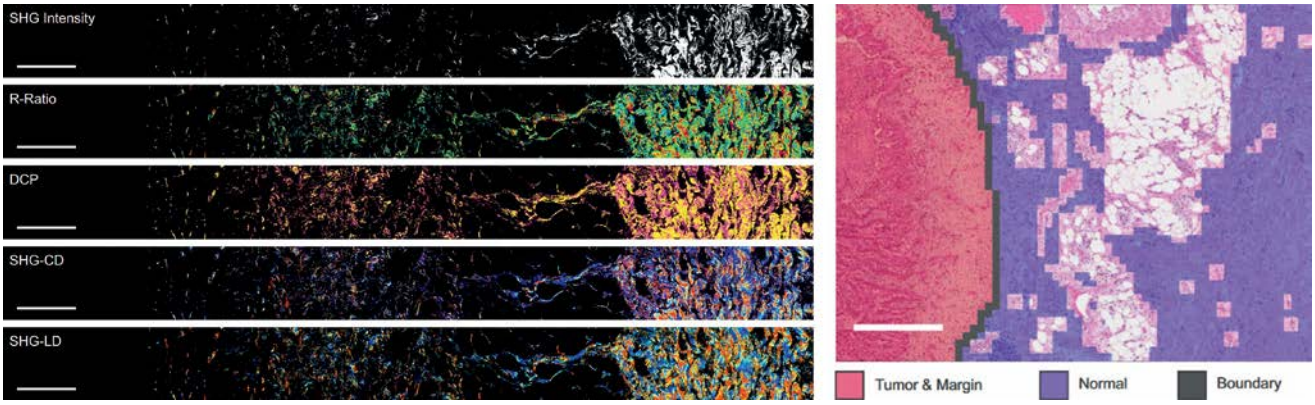


SHG and THG images of H&E-stained colon using FLINT femtosecond oscillator.

Courtesy of Virgis Barzda group, Vilnius University.

WIDEFELD POLARIMETRIC SHG MICROSCOPY

Cancer diagnosis and surgical treatment that rely on imaging require specificity and high throughput. Polarization-resolved second-harmonic generation (P-SHG) microscopy shows potential in visualizing structural changes in collagen networks and the extracellular matrix accompanying tumor development. Furthermore, P-SHG is label-free and compatible with live tissue imaging at depth. However, traditional raster-scanning is too slow for clinical applications, and the structural sensitivity of P-SHG is often hard to interpret. Nonlinear widefield microscopy utilizes amplified femtosecond lasers to increase imaging throughput and the field of view, while machine learning (ML) enables data-driven analysis that can, for example, automate tumor margin delineation and scoring. PHAROS and CARBIDE lasers, in conjunction with ML-augmented widefield microscopy, have the potential to bring the benefits of nonlinear microscopy to the scale required for biomedical and clinical applications.

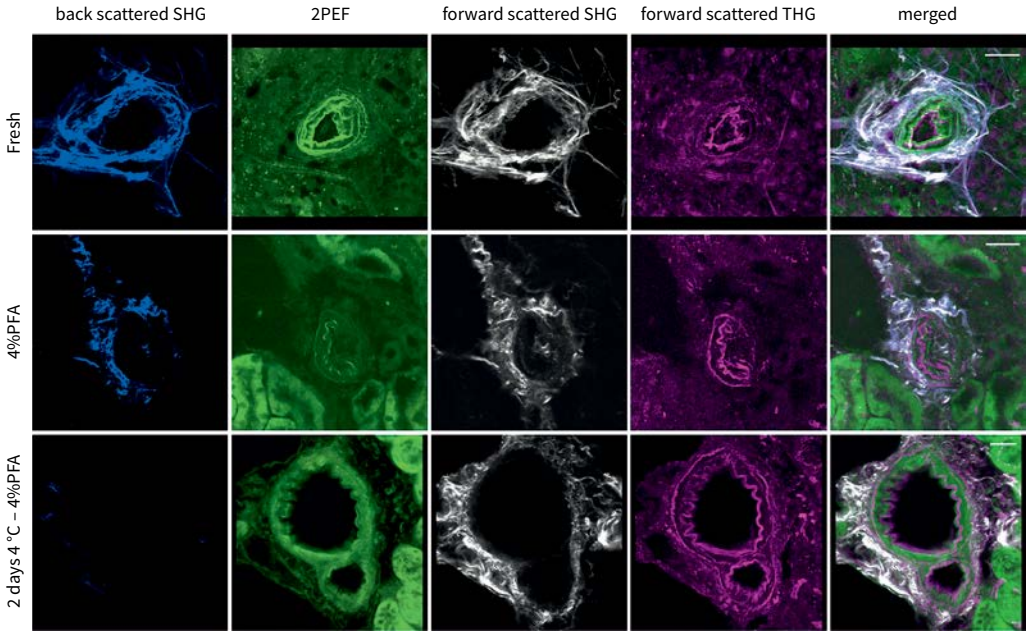


Large-area widefield polarization-resolved SHG microscopy of human lung tissue tumor margin using the PHAROS laser. Image parameters such as SHG intensity, R-ratio, and degree of circular polarization, as well as SHG circular and linear dichroism, are used in unsupervised machine learning to determine the tumor boundary.

Courtesy of Virginijus Barzda group, University of Toronto, and Brian C. Wilson group, Princess Margaret Cancer Centre. Source: Mirsanaye *et al.*, Unsupervised determination of lung tumor margin with widefield polarimetric second-harmonic generation microscopy, Scientific Reports 12 (2022).

SHG, THG, AND 2P IMAGING

Fixation methods such as formalin are commonly used for the preservation of tissue with the aim of keeping their structure as close as possible to the native condition. However, fixatives chemically interact with tissue molecules and may thus modify their structure. Taking advantage of the second- and third-harmonic generation (SHG and THG) emission capabilities of such components, nonlinear two-photon (2P) microscopy was used to evaluate the effect that preservation methods, such as chemical fixatives, have on the nonlinear capabilities of protein components within mouse tissues.



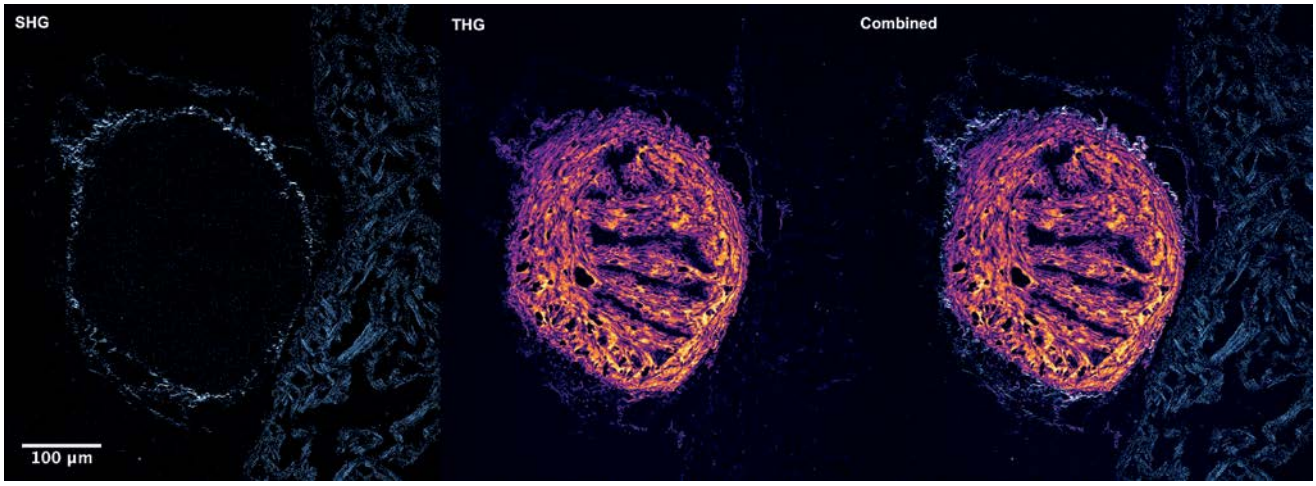
SHG signals from collagen, 2PEF and THG signals from elastin in vibratome sections of mouse kidney after different treatments, using CRONUS-2P femtosecond laser source.

Courtesy of Frauke Alves and Fernanda Ramos-Gomes, Max-Planck Institute for Multidisciplinary Sciences, Germany.
DOI: 10.1364/BOE.488453

COMBINED SHG AND THG IMAGING

Adult zebrafish heart ventricle section from a scar formation study. The brightfield image is stained with Masson's trichrome (MT), connective tissue is blue, muscle is red/brown.

SHG and THG images reveal collagen and muscle structure at the periphery of bulbus arteriosus, while MT-stained elastin is visualized in the center in THG.

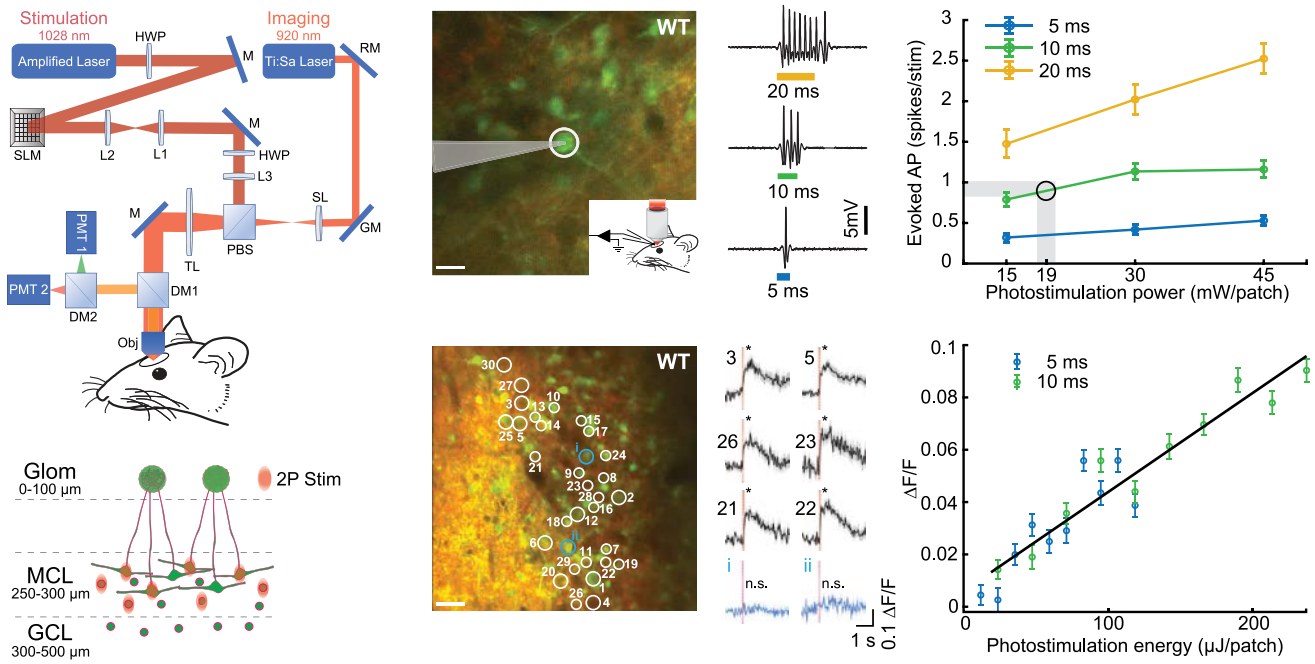


Adult zebrafish heart ventricle section, FLINT femtosecond oscillator used for imaging.

Samples courtesy of Justas Lazutka at the Vilnius University Life Sciences Center. Nonlinear imaging courtesy of the Barzda group at the Vilnius University Department of Physics.

HOLOGRAPHIC 2P OPTOGENETICS

Traditional full-brain neurostimulation using CW light lacks specificity, whereas point-by-point laser-scanning, despite being highly specific, can be slow and is not simultaneous. Holographic multiphoton neurostimulation, on the other hand, is capable of random-access-style volumetric neuron activation and is therefore used for advanced behavioral neuroscience studies. Holographic stimulation is exceptionally demanding on the laser source, requiring very high average power, combined with complex on-demand pulse train control – features that are well supported by the CARBIDE and PHAROS laser families.



Holographic 2P optogenetic stimulation of mouse olfactory bulb neurons using laser system with PHAROS femtosecond laser.

Courtesy of Shy Shoham and Dmitry Rinberg groups, New York University. Source: J. V. Gill *et al.*, Precise holographic manipulation of olfactory circuits reveals coding features determining perceptual detection, Neuron 108 (2020).