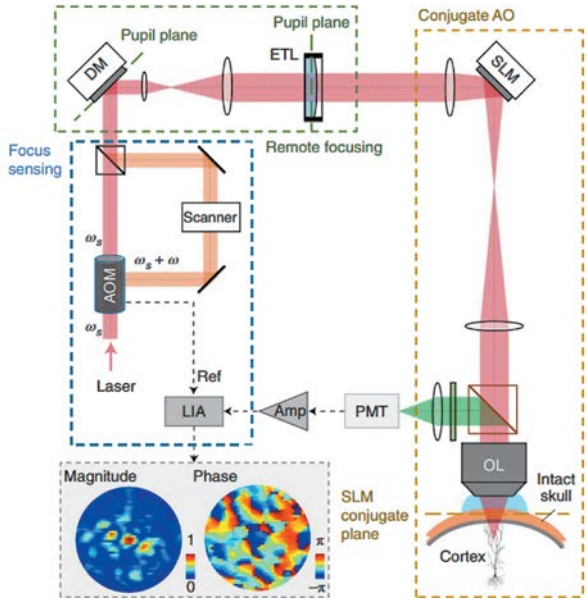
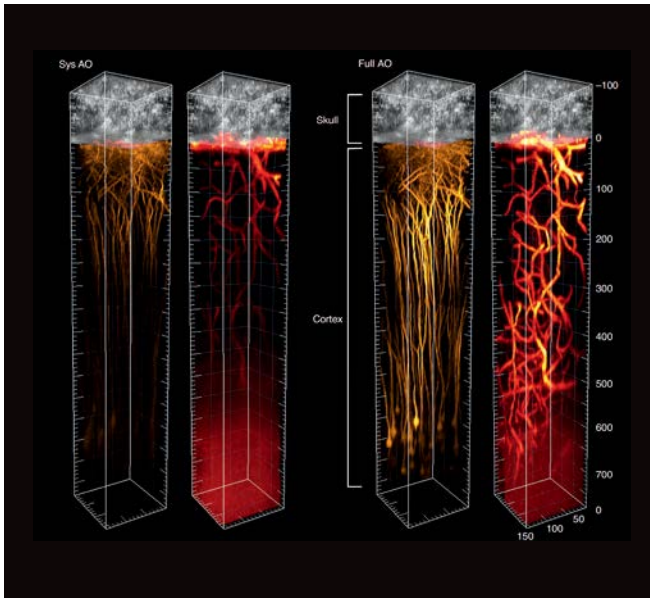
Nonlinear Microscopy

Photonic Solutions Ltd
Edinburgh
EH14 4AP
+44 131 664 8122
www.photonicsolutions.co.uk

Functional 3P neuroimaging

Recording real-time single-neuron activity in the deep brain layers of awake animals is essential for understanding behavior, brain connectivity, and function. These applications have been advanced by neuron imaging and stimulation techniques using high-power, high-pulse-energy lasers with medium-repetition rates, tunable in the SWIR range, which aligns with the biological transparency windows at 1300 nm and 1700 nm. For 2P and 3P excited fluorescence, and harmonic-generation (SHG, THG) imaging in deep tissues, dispersion-controlled femtosecond pulses from **I-OPA** and **ORPHEUS** OPAs and microscopy-dedicated **CRONUS** lasers represent state-of-the-art choices.





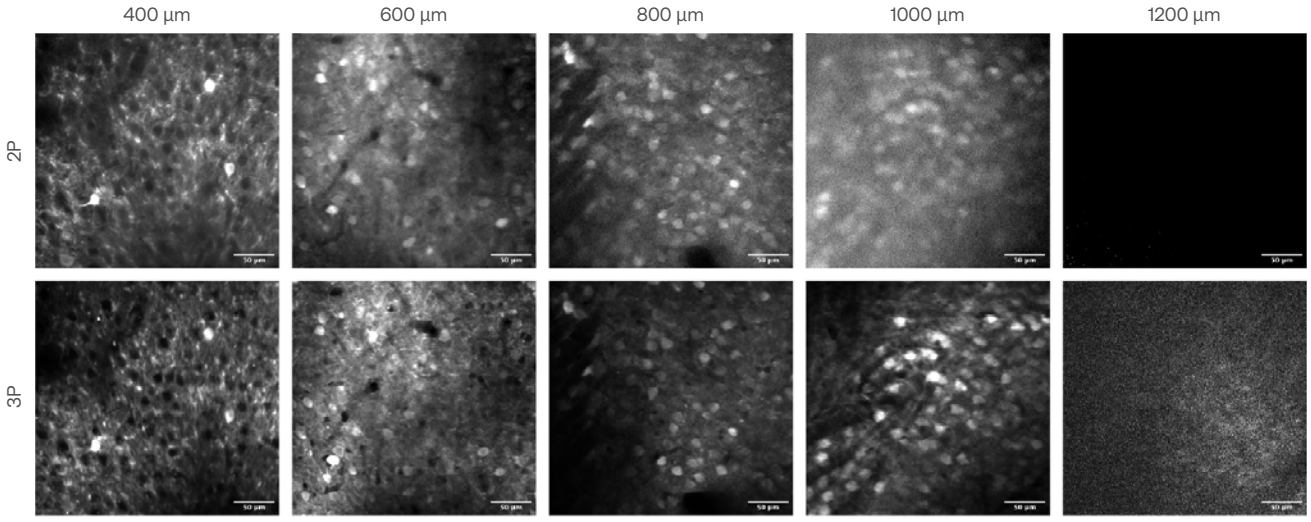
3P microscopy with adaptive optics for focus sensing and shaping to compensate for both aberrations and scattering. **ORPHEUS-F** excitation at 1300 nm enabled imaging up to 1.1 mm below the pia within the intact brain.

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

2P and 3P calcium imaging at depth in mouse brain

Three-photon microscopy (3PM) has gained popularity as a tool able to extend the capabilities of two-photon microscopy (2PM) by imaging deeper layers in the brain and other tissues such as tumors and bone.

Imaging depth in 2PM is limited by the scattering and absorption of excitation light within the tissue. 3PM overcomes this limit because the higher nonlinearity of the 3P excitation reduces the background.

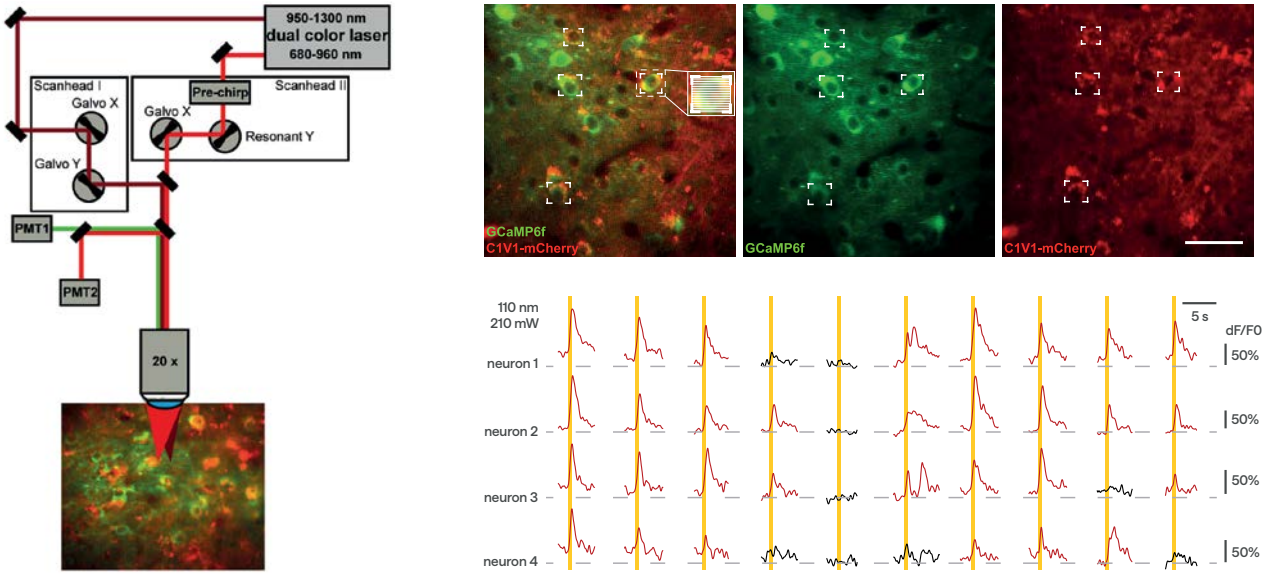


Comparison of in vivo 2P and 3P calcium imaging of mouse visual cortex GCaMP neurons on a Thorlabs Bergamo II microscope using a typical 2P laser and Light Conversion's **CRONUS-3P** (3P) laser at 920 nm and 1300 nm, respectively.

Courtesy of CSHL ISFNS 2024 school organizers, Willis Broden Jr. and Sergey Matveev (Thorlabs).

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, the independent tunability of the two beams enables new coherent Raman scattering modalities.

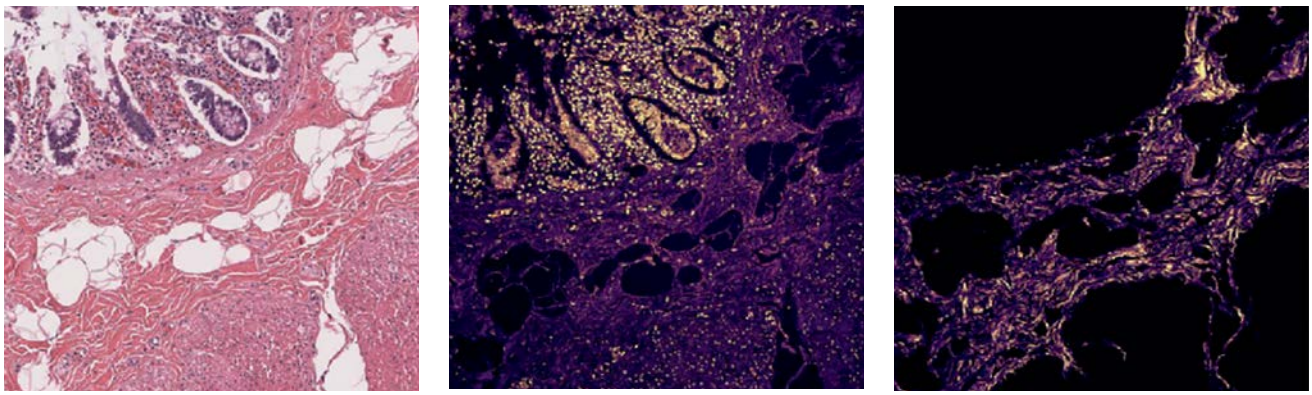


2P optogenetic stimulation of individual neurons using **CRONUS-2P**.

Courtesy of Albrecht 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, 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. The **FLINT** oscillator provides stable 24/7 operation with excellent noise performance, characterized by a RIN of < 140 dBc/Hz above 200 kHz and shot-noise-limited performance at -160 dBc/Hz above 1 MHz.



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

Courtesy of Virginijus Barzda group, Vilnius University.

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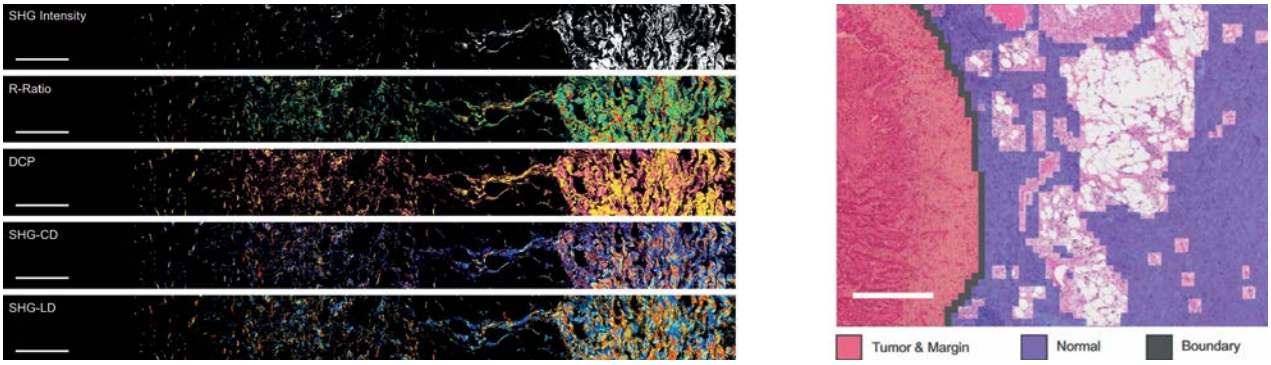
Widefield polarimetric SHG microscopy

Cancer diagnosis and surgical treatment rely on imaging techniques that demand specificity and high throughput. Polarization-resolved second-harmonic generation (P-SHG) microscopy shows potential for visualizing structural changes in collagen networks and the extracellular matrix associated with tumor development. Moreover, P-SHG imaging is label-free and compatible with live tissue imaging at depth. However, traditional raster scanning methods are too slow for clinical applications, and interpreting the structural sensitivity of P-SHG can be challenging.

Nonlinear widefield microscopy addresses these limitations by utilizing amplified femtosecond lasers to increase imaging throughput and field of view. Additionally, machine learning (ML) techniques enable data-driven analysis, facilitating tasks such as automating tumor margin delineation and scoring. By leveraging **PHAROS** and **CARBIDE** lasers in conjunction with ML-augmented widefield microscopy, we can potentially extend the benefits of nonlinear microscopy to the scale required for biomedical and clinical applications.

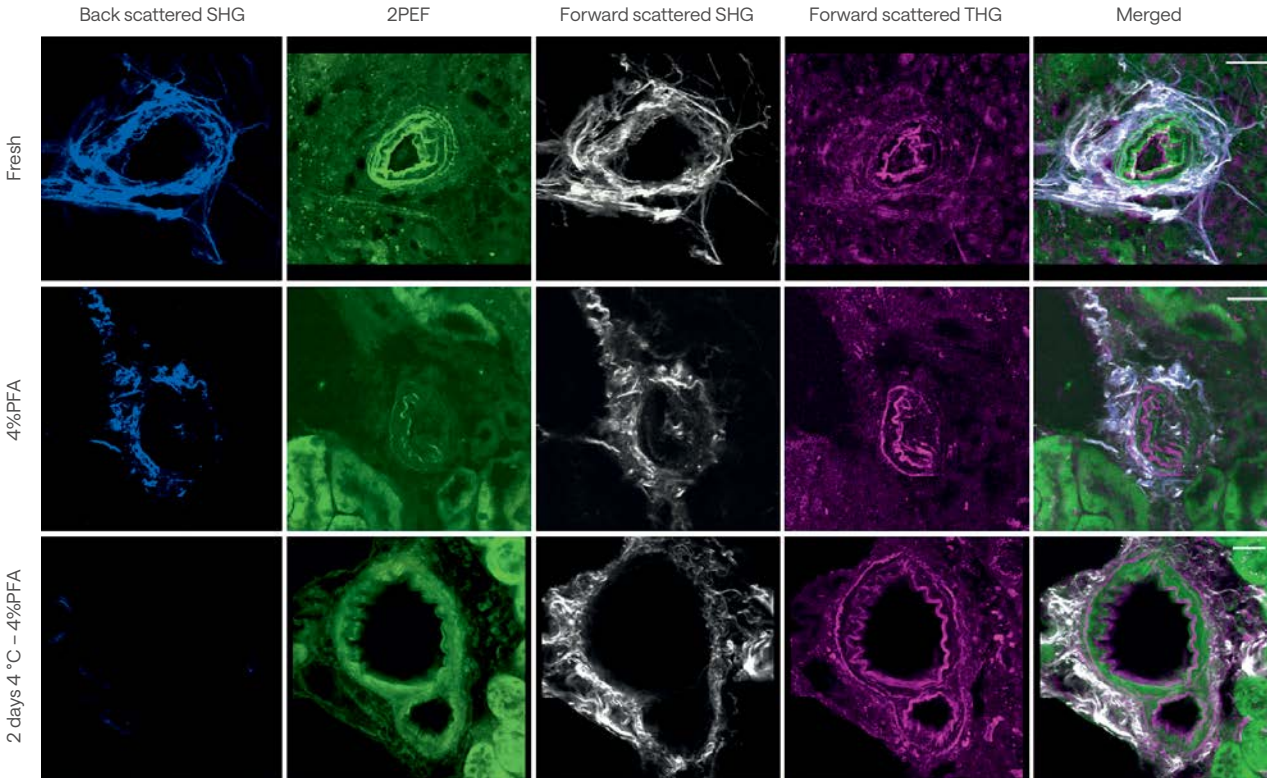


Large-area widefield P-SHG microscopy of human lung tissue tumor margins conducted using the **PHAROS** laser. Image parameters, including SHG intensity, R-ratio, and degree of circular polarization, as well as SHG circular and linear dichroism, are employed in unsupervised ML algorithms to determine 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 tissue preservation to maintain their structure as close as possible to the native condition. However, these fixatives chemically interact with tissue molecules, potentially altering their structure. To assess the impact of preservation methods, such as chemical fixatives, on the nonlinear capabilities of protein components within mouse tissues, nonlinear two-photon (2P) microscopy and the **CRONUS-2P** femtosecond laser were utilized. These techniques take advantage of the SHG and THG emission properties of tissue components.



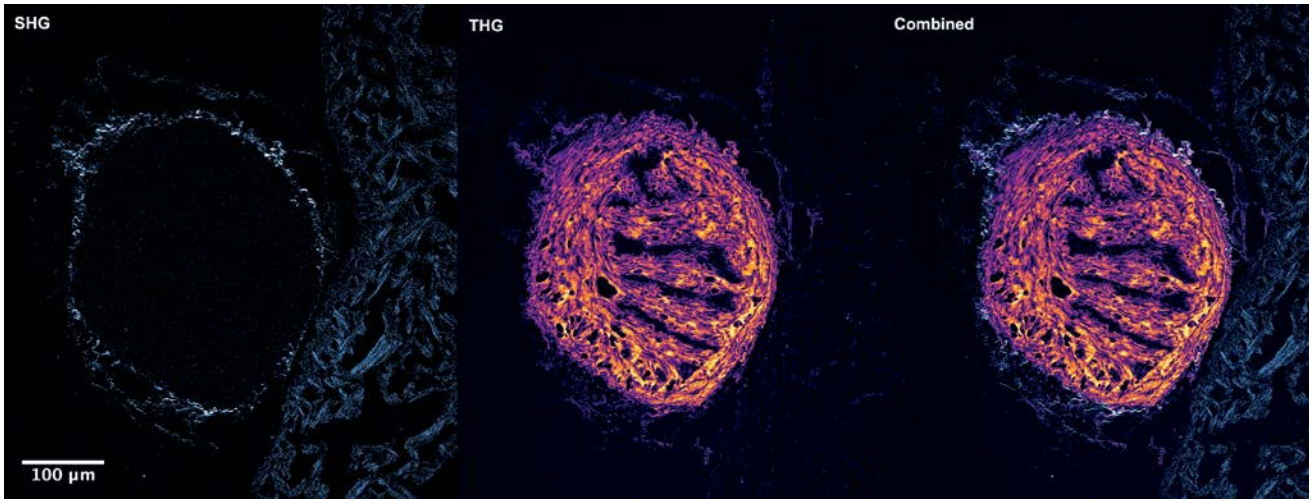
SHG signals from collagen, 2P excitation microscopy and THG signals from elastin in vibratome sections of mouse kidney after different treatments, registered using the **CRONUS-2P** femtosecond laser source.

Courtesy of Frauke Alves and Fernanda Ramos-Gomes, Max-Planck Institute for Multidisciplinary Sciences, Germany.

Combined SHG and THG imaging

Adult zebrafish heart ventricle section used in a scar formation study imaged with the **FLINT** femtosecond oscillator. The brightfield image is stained with Masson's trichrome (MT), where connective tissue appears blue and muscle appears red/brown.

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



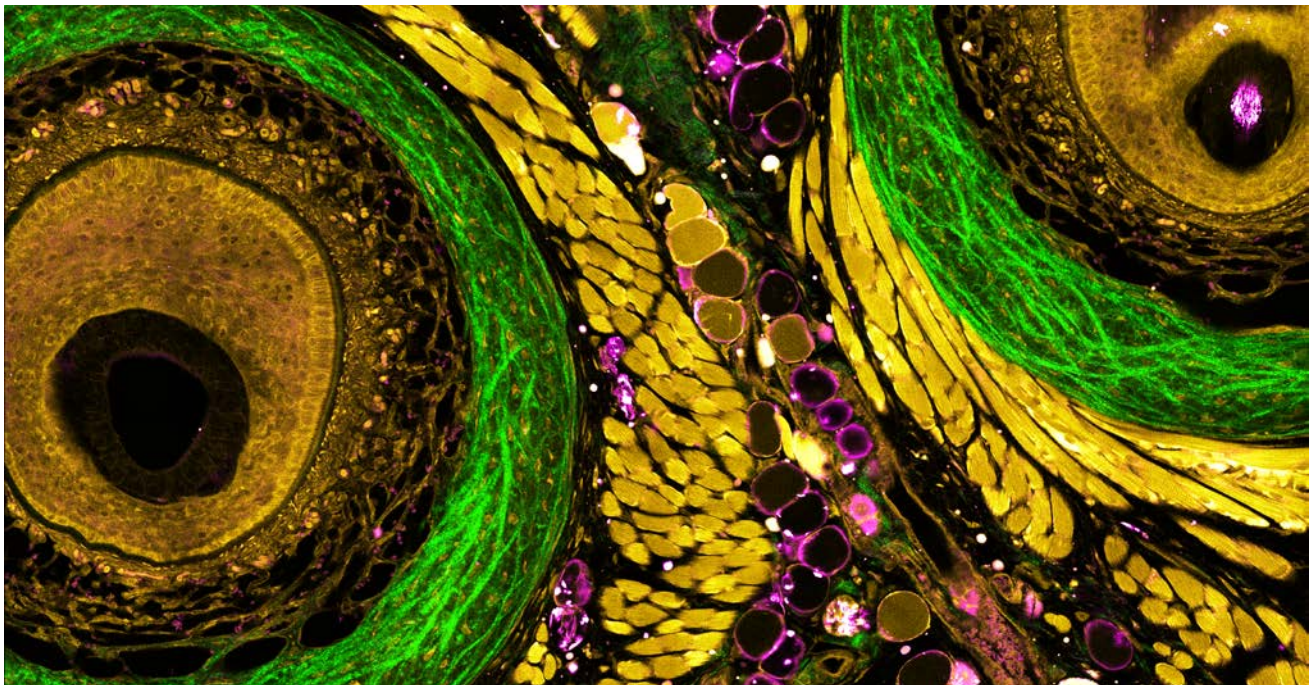
Adult zebrafish heart ventricle section imaged using the **FLINT** femtosecond oscillator.

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

Label-free in vivo imaging

Understanding biological complexity requires minimally disruptive imaging tools capable of providing multiplexed molecular contrasts. To address this need, S. You's laboratory at the Massachusetts Institute of Technology is developing a non-invasive, label-free microscopy approach using CRONUS-3P to visualize biosystems.

As part of a study on neuropathic pain, the image reveals the rich microenvironment of an unprocessed, intact mouse whisker pad: collagen capsule (green), comprising the follicle with muscles (yellow) supporting it, adipocytes (purple), stromal cells, and immune cells.



Mouse whisker pad using label-free microscopy.

Courtesy of Sixian You group, Massachusetts Institute of Technology.

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Photonic Solutions Ltd
Edinburgh
EH14 4AP
+44 131 664 8122
www.photonicsolutions.co.uk