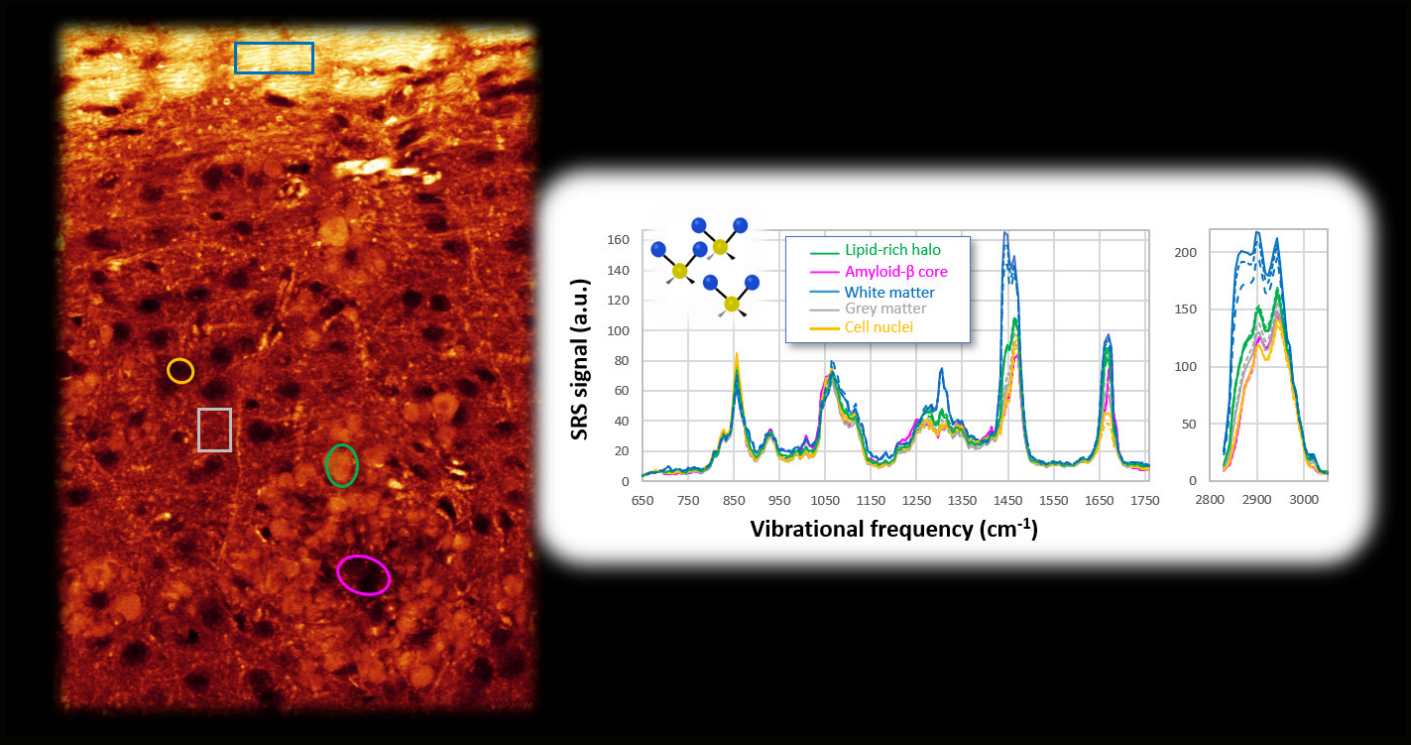
From Eye to Insight

Application Note

TAKING VIBRATIONAL CONTRAST TO THE NEXT LEVEL

STELLARIS 8 CRS



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Chemical Microscopy with Coherent Raman Scattering

Biological samples consist of complex mixtures of molecules. Each type of molecule contains a set of chemical bonds that can vibrate at characteristic vibrational frequencies. Coherent Raman Scattering (CRS) microscopy is the umbrella term for imaging methods that probe these vibrational modes of molecules using laser light. In this way, CRS provides a rich, chemically specific image contrast that arises directly from the endogenous molecules of the sample. No exogenous labels are required, in contrast to fluorescence microscopy. The two most important CRS techniques – Coherent Anti-Stokes Raman Scattering (CARS) and Stimulated Raman Scattering (SRS) – both enable label-free, up to video-rate imaging in living cells, tissues, and even deep inside intact model organisms. While CARS is best known for its great ability to provide crisp, high-resolution images of abundant species, SRS is becoming incresingly popular as a powerful quantiative chemical imaging technique.

Application areas:

> Quantitative spectroscopic imaging of chemically and morphologically complex samples: From cell and tissue biology to medical research, materials science, and microfabrication.

> Probing 3D cellular model systems (organoids) and tissues for preclinical & translational research in cancer, neurodegenerative diseases, immunology, and digestive disorders.

> Multi-modal nonlinear optical microscopy, combining CARS, SRS, Second-Harmonic Generation, and 2-Photon Fluorescence for advanced tissue-based diagnostics.

> Monitoring tissue penetration of pharmacological and cosmetical compounds.

> Quality control in pharma formulations, food samples, chemical products

Label-free characterization of healthy and pathological structures in brain tissues using Stimulated Raman Scattering (SRS). Left: SRS image of lipid structures (2850 cm^{-1}). Right: SRS spectra of regions of interest shown on the left. Sample provided by Dr. Martin Fuhrmann and Andrea Baral, German Center for Neurodegenerative Diseases, Bonn.

Coherent Raman Scattering – Vibrational Imaging with a Boost

Spontaneous Raman scattering – the inelastic scattering of light that results when molecular vibration is induced – has been very successful in providing information on the chemical composition of samples from biology to materials science. However, Raman scattering is a very weak effect and, hence, signal acquisition is known to be painfully slow when used as a microscopy technique. To overcome these limitations, Coherent Raman Scattering Microscopy has been developed as a powerful method to boost the Raman effect. Two laser beams, a Pump laser and a Stokes laser, are focused onto a sample. When the frequency difference between the Pump and Stokes lasers is tuned to exactly match a molecular bond vibration, i.e. $f_P - f_S = f_{vib}$, then the combined action of both beams causes the entire ensemble of molecules to vibrate! This condition is called "Vibrational resonance".

The energy diagrams (Fig. 1) show how the Pump and Stokes beams interact with the molecules. In **Stimulated Raman Scattering (SRS)** [1], the absorption of a Pump photon to a virtual state and the simultaneous emission of a Stokes photon leave the molecule vibrating. As a consequence, whenever the frequency difference between the Pump and Stokes beams hits a molecular vibrational frequency, there is a redistribution of photons between the two beams that can be detected and provides a chemically specific contrast for SRS imaging!

Coherent Anti-Stokes Raman Scattering (CARS) [2] is a related process. The first two steps are the same as for SRS. However, the molecules can absorb yet another Pump photon, and relax by emitting a blue-shifted "Anti-Stokes" photon (Fig. 1). These Anti-Stokes photons are detected using photomultiplier tubes or HyD detectors.

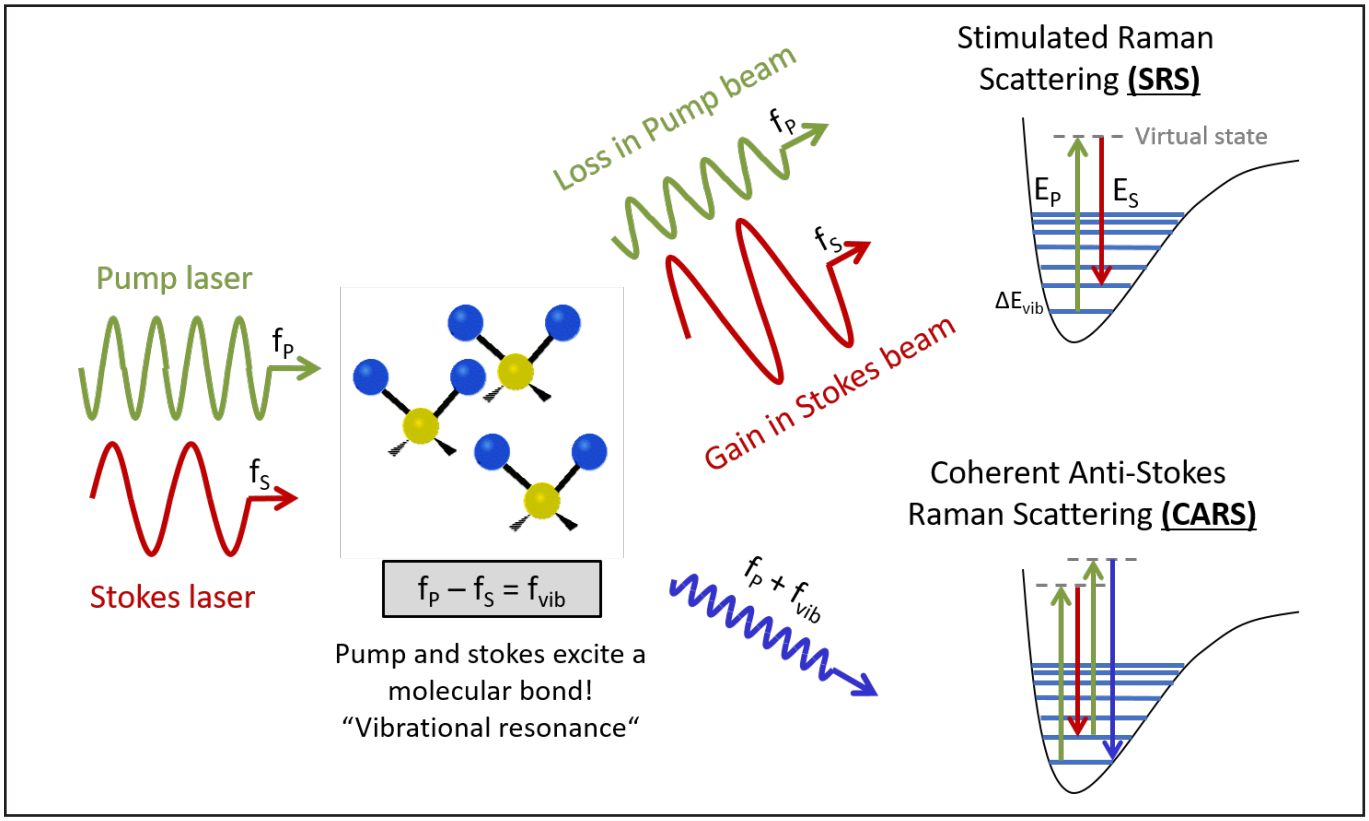


Fig. 1. The SRS and CARS processes occur when the frequency difference between two lasers (Pump and Stokes) matches the vibrational frequency of a molecular bond. Then, the combined action of both beams causes the sample molecules to vibrate. SRS microscopy detects a corresponding loss of photons in the pump beam, while in CARS the emission of blue-shifted Anti-Stokes photons is recorded.

CARS is known for its great ability to provide crisp, high-resolution, label-free images of many different chemical species, such as lipids. More recently, SRS is becoming increasingly popular as a quantitative technique with an enhanced spectroscopic information content that can provide spatial concentration maps of the major chemical components of a sample. In particular, SRS spectroscopic imaging – i.e., the sequential acquisition of SRS images over a range of vibrational frequencies, provides a wealth of information from complex samples, such as biological cells or tissues. For example, lipids, proteins, and aqueous structures are easily distinguished by their spectra. Going further, certain lipid classes (e.g., esters, sterols, phospholipids, saturated vs unsaturated lipids) can be distinguished and certain physical states identified (e.g., misfolding of proteins, ordered vs disordered phases of lipids, different crystal structures). The range of applications is growing almost every day [3-6]!

The STELLARIS 8 CRS microscope offers the flexibility and robustness of Leica Microsystems' all-new STELLARIS confocal platform with the addition of a fully-integrated, turnkey CARS-SRS solution. Additional nonlinear optical signals, such as second harmonic generation (SHG) or multi-photon fluorescence, can be recorded in parallel, providing you with an extremely versatile multimodal optical imaging platform for even your most challenging imaging applications!

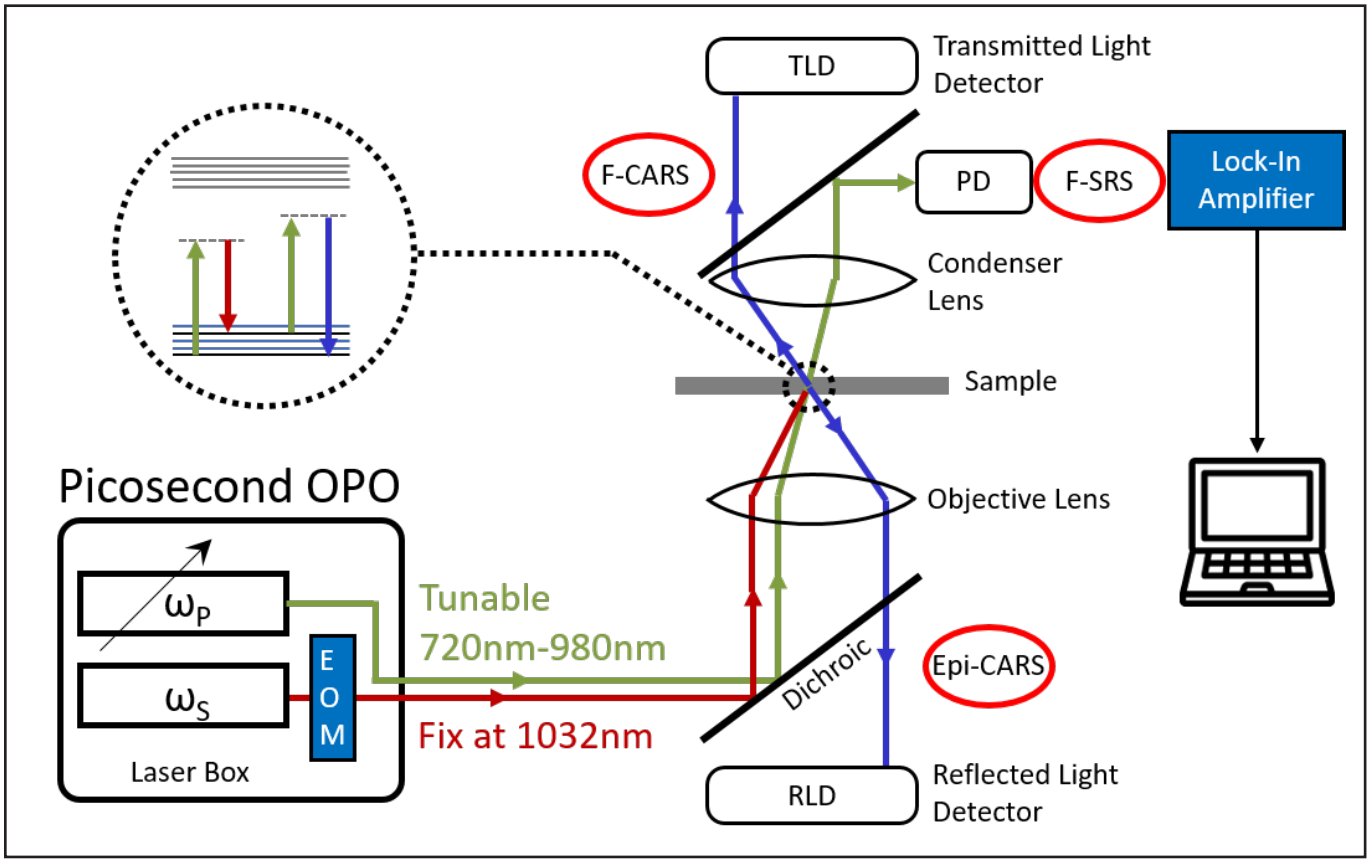


Fig. 2. Beam routing of the STELLARIS 8 CRS microscope. The tunable-frequency Pump beam and the fixed Stokes beam are overlapped in space and time and focused onto the sample. CARS detection is available in the forward and epi directions (F-CARS, Epi-CARS, respectively). For SRS microscopy, the Stokes laser intensity is modulated using an electro-optical modulator (EOM) and the resulting loss of photons in the pump beam is detected in the forward direction using a sensitive photodiode (PD) and a Lock-In amplifier.

Applications – Three-Dimensional Chemical Imaging of Biological Tissues with CARS

A fast and reliable characterization of complex biological samples, such as healthy and pathological tissues, is a hot topic in biology, medicine, and pharma research. Chemically-selective CARS imaging enables a rapid, label-free, high-resolution, three dimensional (3D) visualization of tissues from the macroscopic scale down to sub-cellular structures. A facile discrimination of blood vessels, extracellular matrix components, and cellular assemblies provides a rapid assessment of functional tissue organization.

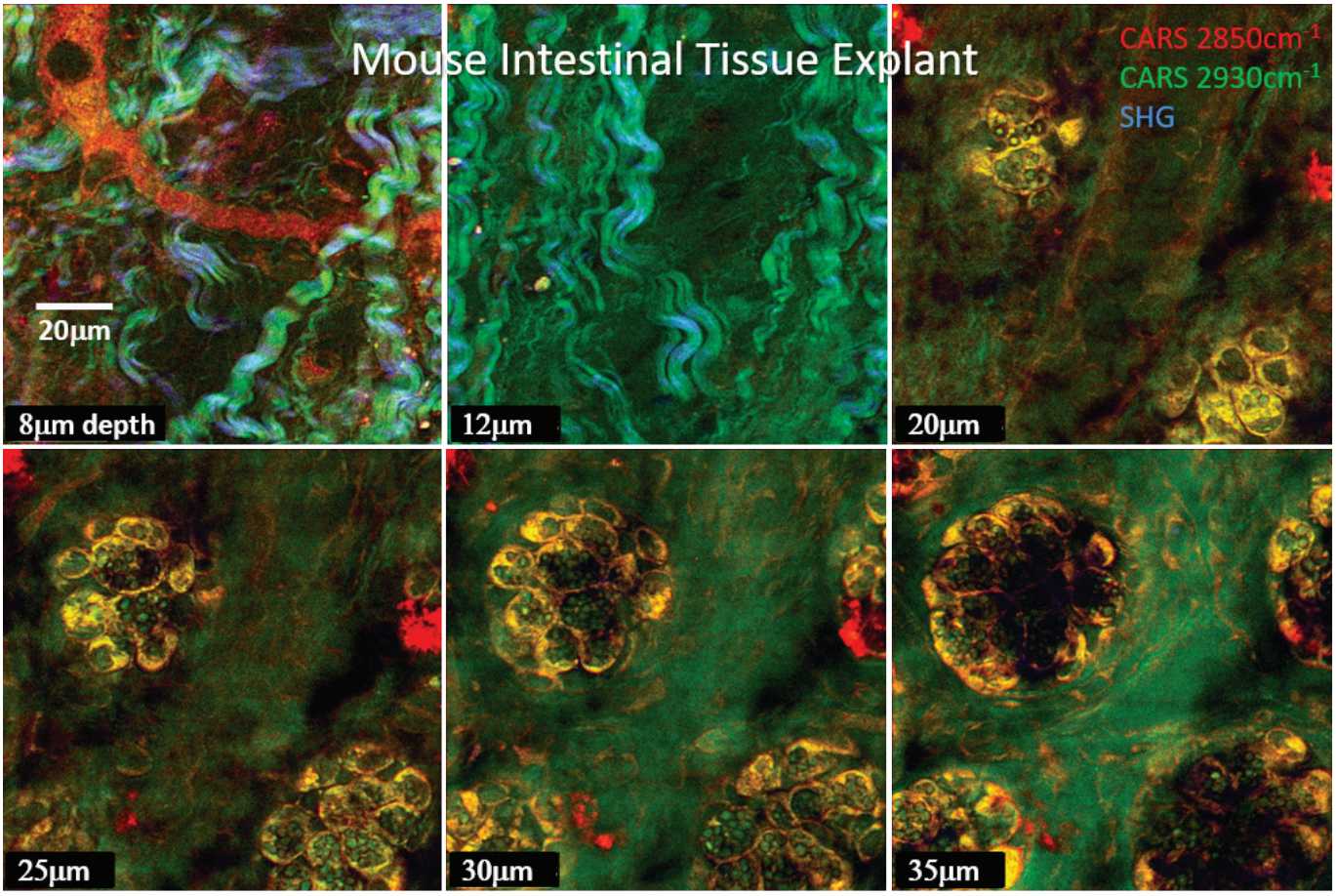
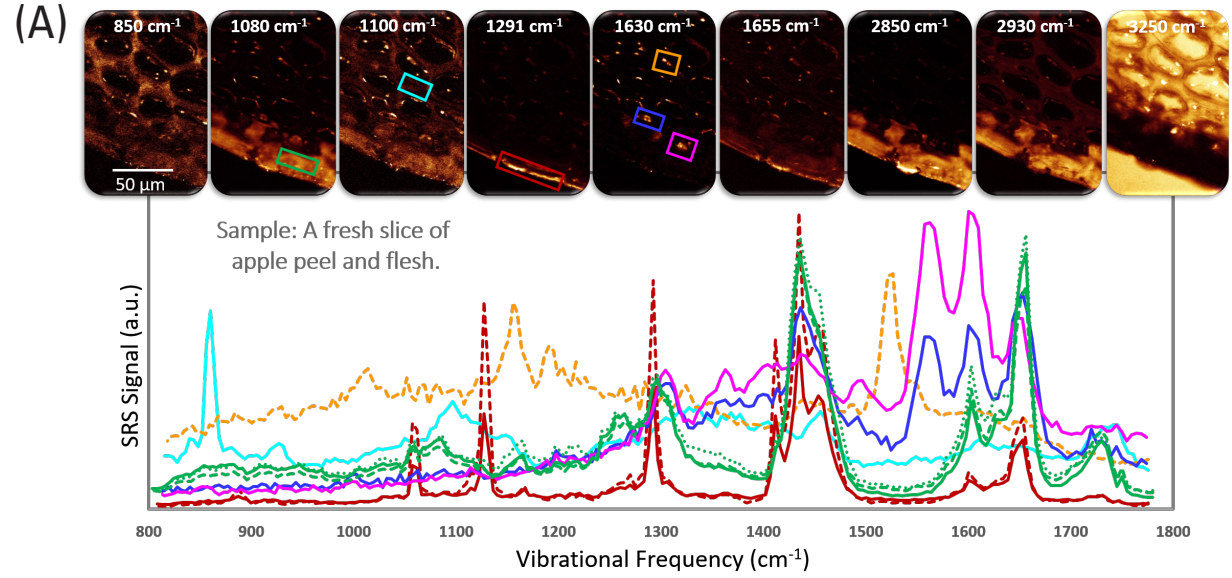


Fig. 3. Two-color CARS and Second-Harmonc Generation (SHG, blue) images of mouse intestinal tissues at different depths show the three-dimensional tissue organization. Green (red) colors indicate chemical contrast of protein-rich and lipid-rich structures, respectively. At 8-12 µm depth, collagen fibers (blue-green) and blood vessels (red) are found, whereas intestinal crypts (yellow/green) are found in deeper layers. Visible in the crypts are the lipid-rich membranes (yellow) and protein-rich granules (green) of paneth cells.

Applications - Chemical spectroscopic imaging with SRS

With the unrivalled speed gains of SRS over spontaneous Raman spectroscopy, chemically spectroscopic imaging with high-resolution and large fields-of-view is now becoming a reality for the first time. A typical hyperspectral SRS imaging workflow is illustrated in Figure 4 A. SRS images at different vibrational frequencies are recorded sequentially by tuning the Pump laser frequency. The spectra of the highlighted regions of interest are shown below. Spectral unmixing algorithms are available to visualize sample structures with distinct biochemical compositions (Figure 4 B). Furthermore, since the SRS spectra are mathematically equivalent to spontaneous Raman spectra, users can tap into the large arsenal of quantitative chemical analysis procedures that have been developed by the Raman spectroscopy community.



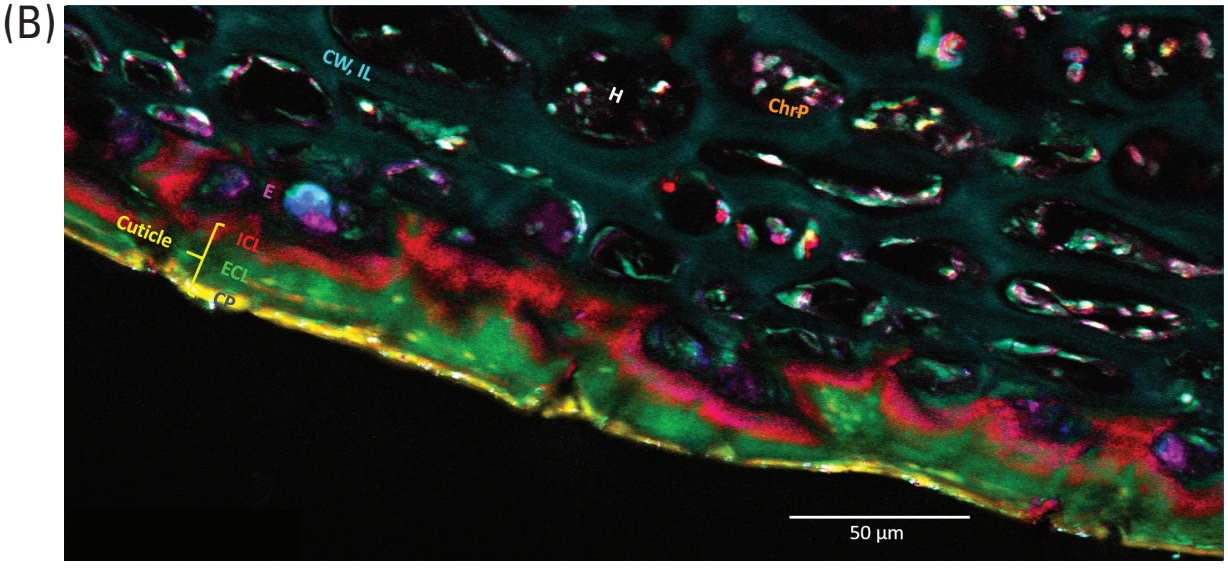


Fig 4: (A) Illustration of SRS spectroscopic imaging with a fresh apple slice. SRS images are recorded sequentially, and the spectra of the highlighted regions of interest are shown below, revealing the amazing biochemical complexity of this sample. (B) A spectral separation based on the spectra recorded in (A) reveals the different cuticular layers of the peel (Cuticle Proper, CP; External and internal cuticular layer, ECL and ICL), and structures of the fruit flesh including epidermal cells (E), cell walls (CW), inner lamina (IL), and chromoplasts (ChrP).

Applications – Probing Tissue Biology and Pathology Without the Need for Staining

Alzheimer's disease (AD) is a chronic neurodegenerative disease in which Amyloid-ß (Aß) plaques appear in affected brain tissues. Recent hypotheses suggest that certain classes of lipids may play a role in the progression of AD by affecting the transport of toxic Aß species into brain tissues. Here, we demonstrate that SRS mircoscopy is uniquely suited to investigate the interplay between Aß and lipid species [7].

In particular, a frequency-shift of the Amide I mode allows the clean and specific visualization of pathological aggregated Aß amidst the healthy protein content of the brain. Furthermore, SRS spectroscopic imaging was used to probe the distributions of several lipid classes, revealing an enrichment of membrane phospholipids in plaque-associated lipid deposits, while cholesterol was found predominantly in healthy white matter structures without much specific localization to plaques. These results highlight the potential of SRS to contribute more broadly to a deeper understanding of neurodegenerative diseases.

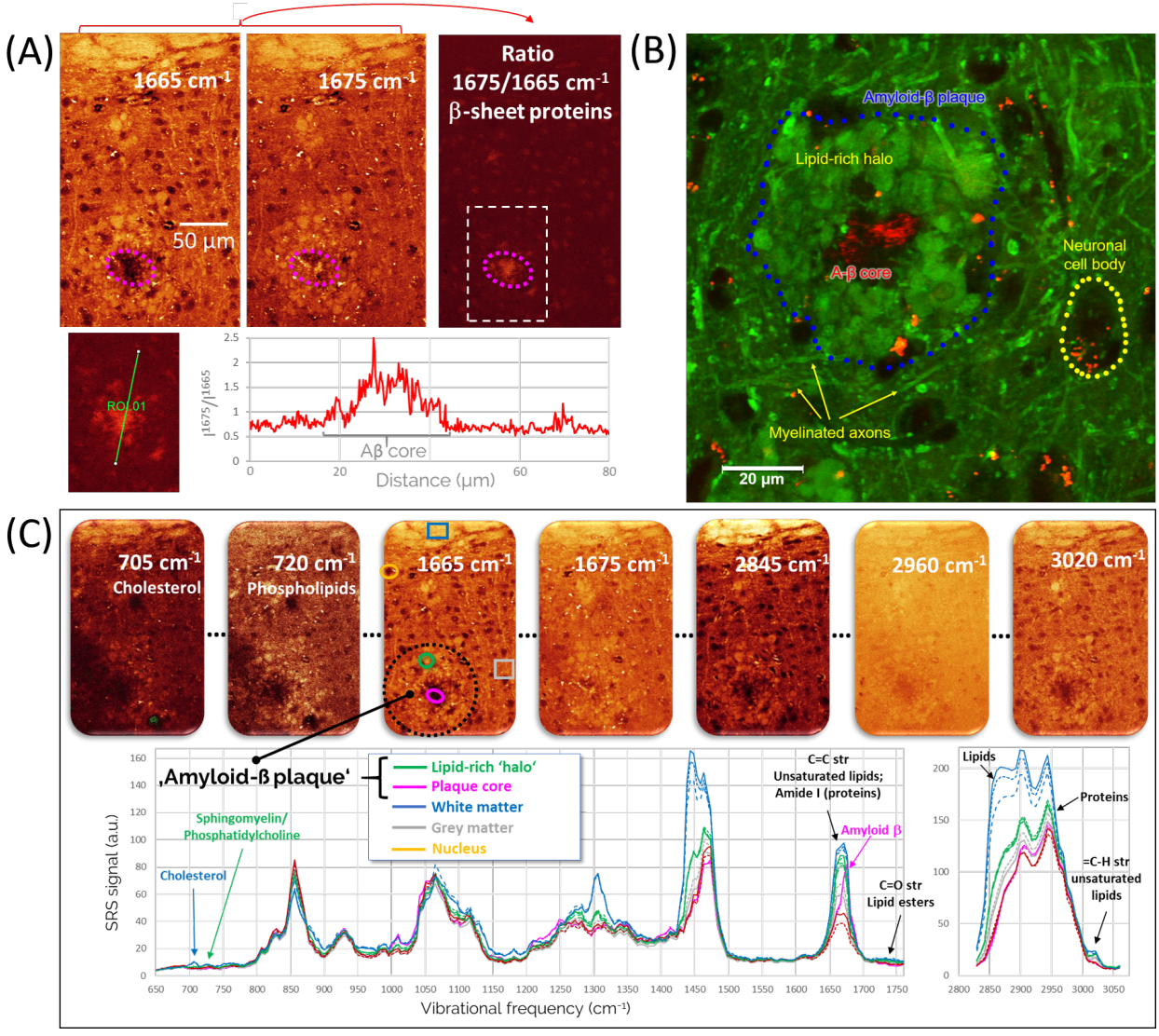


Fig. 5. Label-free spectroscopic imaging of mouse brain slices with SRS. (A) A ratiometric two-color SRS procedure provides a clean visualization of misfolded Amyloid-ß (Aß) plaques. (B) SRS image of lipids (green) and Aß (red) reveals that plaque cores are surrounded by prominent lipid-rich deposits. (C) SRS spectroscopic imaging provides a biochemical characterization of healthy and pathological structures. For example, cholesterol is found largely in the healthy white matter regions (705 cm^{-1}, top of the image), whereas membrane phospholipids are enriched in Aß plaque-associated deposits (720 cm^{-1}, bottom).

Applications – Multimodal Morphochemical Imaging Inside Intact Model Organisms

Using Coherent Raman Scattering, researchers can probe physiological structures nondestructively, even deep inside an intact organism. Tissues and organs are visualized with a reliable sub-cellular resolution and rich contrast based on morphology and biochemical composition. Combine this morphochemical imaging with the additional signal modalities, Second-Harmonic Generation (SHG) and two-photon-excited autofluorescence (2PAF), and you realize the full potential of this unique multimodal imaging platform .

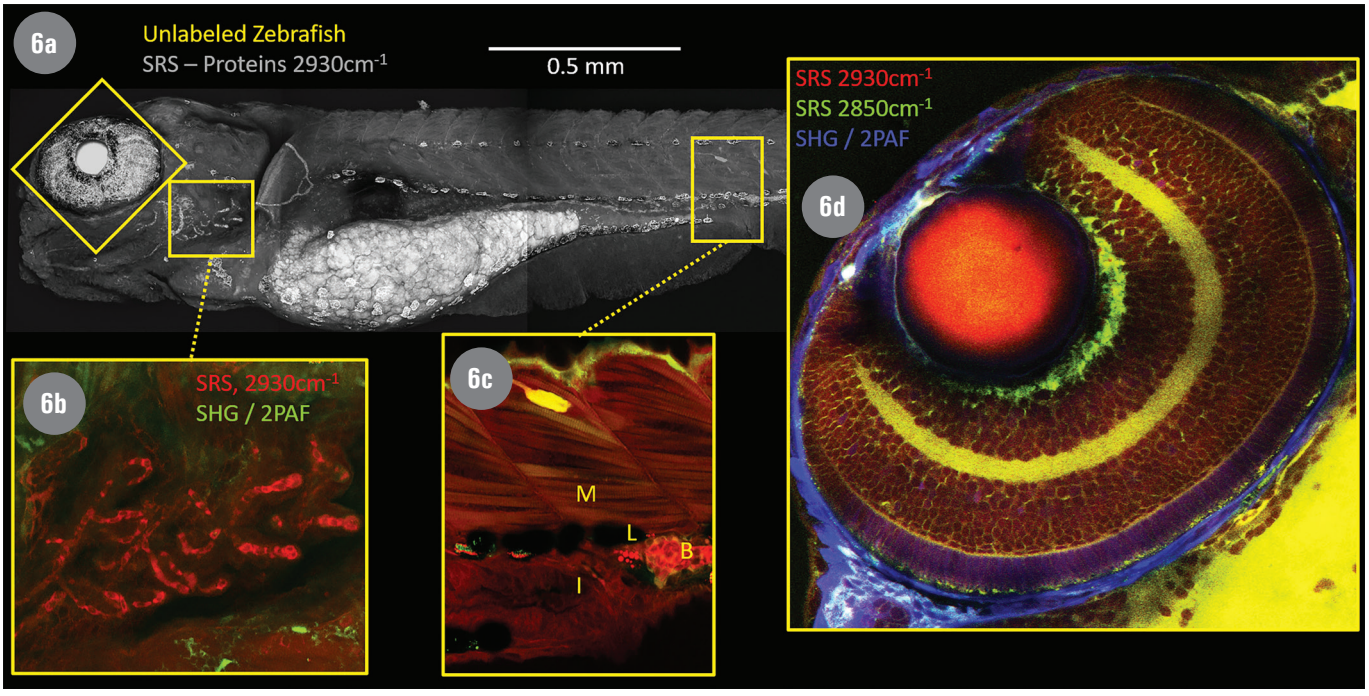


Fig. 6. (A) Grey: overview SRS image of the total protein content in an unlabeled, intact Zebrafish. (B) Zoom-in of the vasculature showing individual blood cells and two-photon excited autofluorescence (2PAF) from surrounding tissues. (C) Zoom-in of the tail region. M: striated muscle tissue; I: intestine; B: blood cell precursors in the posterior blood island; and L: lipid droplets. (D) Multicolor-SRS image of the intact eye showing the different layers of the retina at cellular resolution with contrast provided by proteins (red), lipids (green), and second-harmonic generation (SHG) signals from the outer layer of the eye (sclera).

Probing Organoids for Applications From Cell Biology to Disease Research

Organoids are in vitro-grown 3D cell assemblies that capture some of the key multicellular, anatomical, and functional aspects of real organs. They have a vast potential for applications ranging from basic and preclinical research to drug discovery and personalized medicine.

The STELLARIS 8 CRS provides a versatile imaging toolbox for probing these 3D model systems from the macroscopic scale down to deep-sub-cellular details. By combining confocal imaging of fluorescent markers and CRS imaging in one platform, the activities of molecular pathways can be studied while monitoring the resulting downstream biochemical, metabolic, and structural alterations of the organoids.

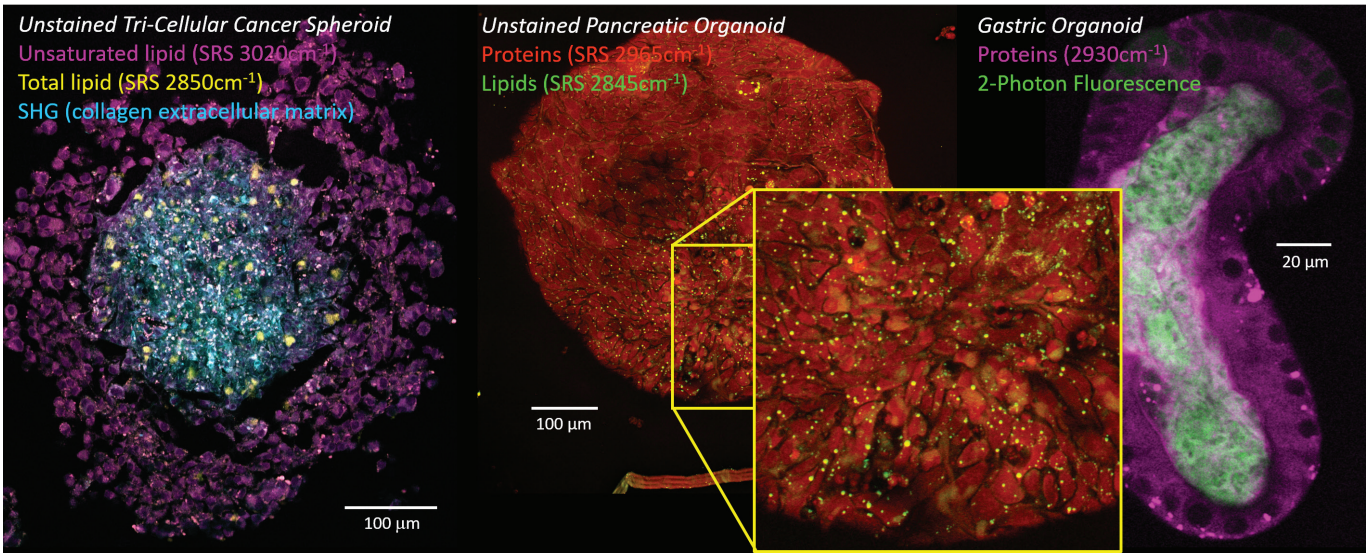


Fig. 7. Label-free imaging of 3D cellular models from cancer research to developmental biology. (Right) The combination of two-color SRS and second-harmonic generation (SHG) images provides biochemical and biophysical contrast to visualize distinct cell types and phenotypes in a tri-cellular spheroid for cancer research. (Middle) Two-color SRS reveals the sub-cellular localization of proteins and lipid vesicles (potential precursors to insulin secretory granules) in an intact pancreatic organoid. (Left) SRS and 2-photon-fluorescence image of an intact gastic organoid revealing the established epithelial tissue architecture and cellular polarization, as well as autofluorescent intraluminal deposits.

Citations

[1] Video-rate molecular imaging in vivo with stimulated Raman scattering. Saar BG, Freudiger CW, Reichman J, Stanley CM, Holtom GR, Xie XS. Science. 2010 Dec 3;330(6009):1368-70. DOI: 10.1126/science.1197236.

[2] Vibrational Microscopy Using Coherent Anti-Stokes Raman Scattering. Zumbusch A, Holtom GR, Xie XS. Phys Rev Lett 82 (1999) 4142. DOI: 10.1103/PhysRevLett.82.4142

[3] Vibrational spectroscopic imaging of living systems: An emerging platform for biology and medicine. Cheng JX, Xie XS. Science. 2015 Nov 27;350(6264):aaa8870. DOI: 10.1126/science.aaa8870.

[4] Coherent Raman Scattering Microscopy in Biology and Medicine. Zhang C, Zhang D, Cheng JX. Annu Rev Biomed Eng. 2015;17:415-45, Epub 2015 Oct 22. DOI: 10.1146/annurev-bioeng-071114-040554.

[5] Further recommended reading can be found in Leica Microsystem's microscopy knowledge portal Science Lab: https://www.leica-microsystems.com/science-lab/cars-publication-list/

[6] Biological imaging of chemical bonds by stimulated Raman scattering microscopy. Hu Fanghao, Shi Lixue, Min Wei, Nature Methods. 2019, Aug 30. 16, 830-842.

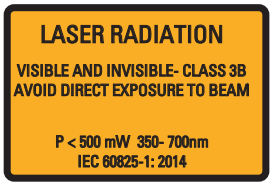
[7] Label-free characterization of Amyloid-ß-plaques and associated lipids in brain tissues using stimulated Raman scattering microscopy. Schweikhard V, Baral A, Krishnamachari V, Hay WC, Fuhrmann M, bioRxiv 789248 2019, doi:10.1101/789248

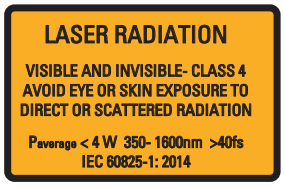
Specifications: STELLARIS 8 CRS

Leica Microsystems offers a fully-integrated Coherent Raman Scattering (CARS/SRS) solution which is built on its all-new STELLARIS confocal laser scanning platform.

Key Features:

- CRS laser picoEmerald S from APE:
  - Pump laser: Optical Parametric Oscillator (OPO) – tunable from 720 nm - 980 nm.
  - Stokes laser: Fiber laser, fixed at 1032 nm, frequency doubled to pump the OPO.
  - 20-MHz EOM intensity modulation of the Pump laser for SRS.
- CRS optimized Beam Routing.
- Dedicated IR objectives for optimized CRS signal generation and detection.
- Truly multi-modal nonlinear optical microscopy with 2-Channel Detector Option: Simultaneous detection of CARS and Second-Harmonic Generation (SHG)/2-Photon Fluorescence signals (available for Forward and Epi detection).
- Switch between Forward SRS and Forward-CARS detection. Simultaneous detection of Epi-CARS signals.
- Accessible range of vibrational frequencies for SRS: 3500 – 507 cm^{-1}, covering the entire high-wavenumber, cell-silent, and fingreprint regions.
- Accessible range of vibrational frequencies for CARS: 3500 – 1200 cm^{-1}.
- Fully integrated and software-controlled SRS Detector + Lock-In Amplifier settings guarantee optimized signal detection for any scan format.





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