In vivo Imaging with Stimulated Raman Scattering Microscopy

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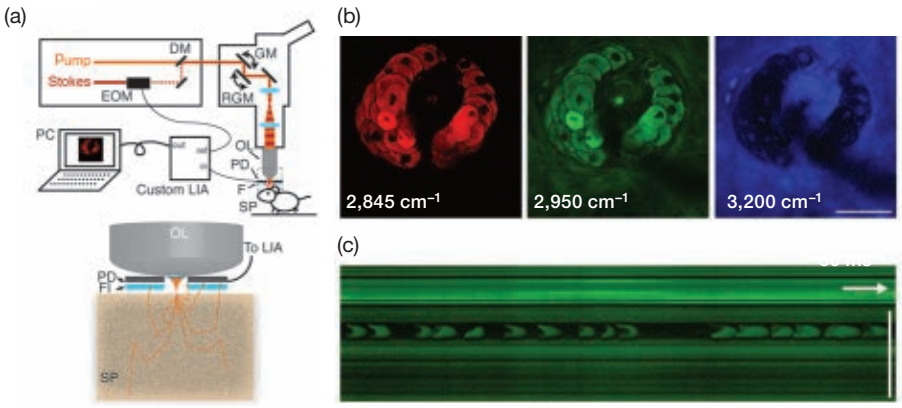
Fluorescence microscopy and genetically encodable fluorophores have transformed biological research and medical diagnostics. Unfortunately, labels can be bothersome, particularly for small-molecule imaging, limiting their use for *in vivo* diagnostics. Vibrational microscopy can provide an alternative. It relies on a label-free chemical contrast based on the intrinsic properties of the sample. However, traditional methods that utilize spontaneous Raman scattering or infrared absorption have limited speed or spatial resolution, respectively.

Coherent Raman scattering (CRS), which includes coherent anti-Stokes Raman scattering (CARS)[1,2] and stimulated Raman scattering (SRS) microscopy, overcomes these limitations.[3,4] The sample interacts with two excitation fields whose difference frequency is tuned to match the target molecule's vibrational frequency. This coherent excitation results in signals up to five orders of magnitude larger than spontaneous Raman scattering, enabling three-dimensional imaging with high temporal resolution.[1-4]

In contrast to CARS, SRS is free from the non-resonant background that causes image artifacts, spectral distortions and limited sensitivity. SRS is also linearly dependent on the number of target molecules and free from coherent image artifacts due to phase matching, allowing quantitative chemical imaging.[3,4]

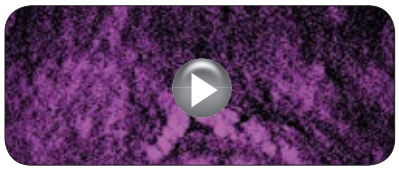
While both phenomena occur simultaneously, they differ greatly in their detection schemes. In CARS, radiation at a new optical frequency is detected. In SRS, the intensity gain and loss (absorption) of the excitation beams must be measured in the presence of laser intensity fluctuations and varying sample transmission during raster-scanning the laser foci across the sample. In 2008, we demonstrated SRS detection under biomedical excitation conditions. By implementing a high-frequency modulation transfer scheme, we achieved close to shot-noise-limited sensitivity.[4] However, the acquisition speed was slow and the imaging of thick, non-transparent samples was limited by inefficient collection of the back-scattered signal.



(a) Pump and Stokes beams are combined with a dichroic mirror and colinearly focused into the specimen. The position is scanned at video-rate (30 frames/s) with galvano mirrors. The Stokes beam is modulated at 20 MHz with an electro-optic modulator and blocked after the sample with a filter. The pump is detected with a photodiode, and the SRS modulation transfer is extracted by a custom lock-in amplifier. We excite through the center aperture of a large-area annular detector for high collection efficiency of the back-scattered signal. (b) Label-free imaging of a mouse sebaceous gland at indicated Raman shifts characteristic for lipids (red), protein (green) and water (blue). (c) *In vivo* SRS flow cytometry performed by acquiring a line-scan across a capillary as a function of time, showing individual red blood cells in a capillary. RBCs are captured without motion. Scale: 20 μm.

We have recently improved the imaging speed to video-rate using home-built wideband detection electronics that support 100 nanosecond pixel dwell times.[5] In tissue samples, signal detection in reflection is possible based on back-scattering of the forward-travelling signal by the sample. This does not compromise the optical sectioning capabilities of SRS because nonlinear signals are only generated at the focus, where intensities are the highest. We performed Monte Carlo simulations to quantitatively understand the light diffusion and designed a large-area annular detector to maximize light collection efficiency. Using this new design, we collected about 30 percent of the forward-travelling signal and enabled *in vivo* imaging in mice and humans without motion blur.[5]

With these recent advances, SRS microscopy has superseded CARS microscopy for chemical imaging in biology and medicine.



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