

Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy

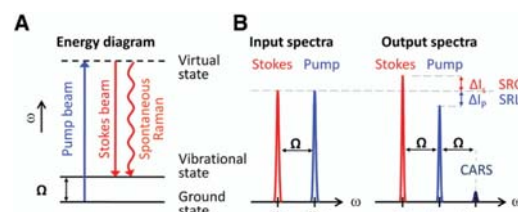
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Label-free chemical contrast is highly desirable in biomedical imaging. Spontaneous Raman microscopy provides specific vibrational signatures of chemical bonds, but is often hindered by low sensitivity. Here we report a three-dimensional multiphoton vibrational imaging technique based on **stimulated Raman scattering (SRS)**. The sensitivity of SRS imaging is significantly greater than that of spontaneous Raman microscopy, which is achieved by implementing high-frequency (megahertz) phase-sensitive detection. SRS microscopy has a major advantage over previous coherent Raman techniques in that it **offers background-free and readily interpretable chemical contrast**. We show a variety of biomedical applications, such as differentiating distributions of omega-3 fatty acids and saturated lipids in living cells, imaging of brain and skin tissues based on intrinsic lipid contrast, and monitoring drug delivery through the epidermis.

Vibrational microscopies based on infrared absorption and **Raman scattering** (1, 2) have been used as **label-free contrast** mechanisms due to characteristic frequencies of various chemical bonds. However, infrared microscopy has limited spatial resolution because of long infrared wavelengths. **Spontaneous Raman scattering microscopy**, while having **higher spatial resolution** due to shorter excitation wavelengths, is **insensitive** and thus often has **limited imaging speed**. Coherent anti-Stokes Raman scattering

(**CARS**) microscopy **offers higher sensitivity** than spontaneous Raman microscopy (3, 4). However, a CARS spectrum is different from its corresponding spontaneous Raman spectrum due to a **nonresonant background**, which **complicates spectral assignment**, causes difficulties in image interpretation, and limits detection sensitivity.

Here we explore **stimulated Raman scattering (SRS)** as an imaging contrast mechanism. SRS is analogous (5, 6) to the well-known phenomenon of stimulated emission (7) and was first observed



in 1962 (8). Since then it has been used in many spectroscopic studies (9–12). In spontaneous Raman scattering, one laser beam at a frequency ω_p illuminates the sample and the signal is generated at the Stokes and anti-Stokes frequencies, ω_s and ω_{AS} , respectively, due to inelastic scattering. In SRS, however, two laser beams at ω_p and ω_s coincide on the sample (Fig. 1A). When the difference frequency, $\Delta\omega = \omega_p - \omega_s$, also called the Raman shift, matches a particular molecular vibrational frequency Ω , amplification of the Raman signal is achieved by virtue of stimulated excitation. Consequently, the intensity of the Stokes beam, I_S , experiences a gain, ΔI_S (**stimulated Raman gain, SRG**), and the intensity of the pump beam, I_P , experiences a loss, ΔI_P (**stimulated Raman loss, SRL**), as shown in Fig. 1B. In contrast, when $\Delta\omega$ does not match any vibrational resonance, SRL and SRG cannot occur. Therefore, **unlike CARS, SRL and SRG do not exhibit a nonresonant background** (11).

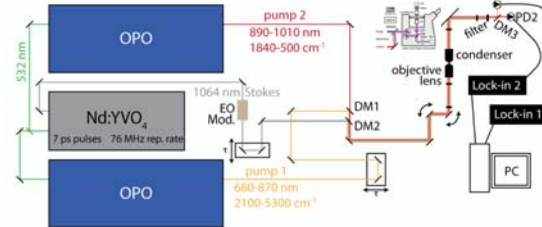
The intensity of SRG or SRL is described by $\Delta I_S \propto N \times \sigma_{\text{Raman}} \times I_P \times I_S$ and $\Delta I_P \propto -N \times$

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$\sigma_{\text{Raman}} \times I_p \times I_s$, where N is the number of molecules in the probe volume and σ_{Raman} is the molecular Raman scattering cross-section (6). As in other multiphoton techniques (3, 13), the **non-linearity of SRL and SRG** in the overall excitation intensity **allows three-dimensional (3D) sectioning**. Such nonlinear excitation is typically accomplished by picosecond or femtosecond pulse trains in the near-infrared region.

Method



We take a different approach, using high-repetition rate (76 MHz) picosecond pulse trains with more than three orders of magnitude lower peak power. The pump beam for SRL is provided by a synchronously pumped, tunable optical parametric oscillator (OPO), and the Stokes beam is provided by a 1064-nm mode-locked Nd:YVO₄ oscillator. A 7-ps pulse width is chosen because its frequency bandwidth offers optimal spectral resolution (3 cm⁻¹). Under this excitation condition, the small SRL and SRG signals ($\Delta I_p/I_p$ and $\Delta I_s/I_s < 10^{-4}$) are buried in the laser noise. Realizing that laser noise occurs primarily at low frequencies, we implement a high-frequency phase-sensitive detection scheme, as previously used in other applications (10, 16, 17). For SRL, we modulate the intensity of the Stokes beam at 1.7 MHz and detect the resulting intensity modulation of the pump beam at the same frequency with a lock-in amplifier (Fig. 1C). Similarly, SRG can be measured by modulating the pump beam and detecting the Stokes beam (18). With this approach, $\Delta I_p/I_p < 10^{-7}$ can be achieved with a 1-s time constant. To acquire images via beam scanning, we used a 300- μ s time constant and a pixel dwell time of 170 μ s. It is difficult to incorporate such phase-sensitive detection at radio frequency (MHz) with a multiplex detector such as a diode array. Our approach can detect intensity changes $\Delta I_p/I_p$ and $\Delta I_s/I_s$ four orders of magnitude more sensitive than in the previous report (14).

Beam geometry

Collinear pump and Stokes beams are focused with a high-numerical aperture (NA) objective (NA = 1.2) onto a common focal spot (Fig. 1D). In SRS, the spatial resolution is diffraction limited and similar to that of two-photon fluorescence. Because SRL and SRG are measured at the same frequencies as those of the input fields, phase matching is automatically fulfilled. This allows deconvolution with a point spread function similar to that of fluorescence microscopy and makes image interpretation simpler than in the case of CARS (19).

To detect the pump or Stokes beams in the forward direction, we used a condenser with an NA = 1.35, which is higher than that of the excitation objective, to minimize spurious back-ground due to cross-phase modulation (20, 21).

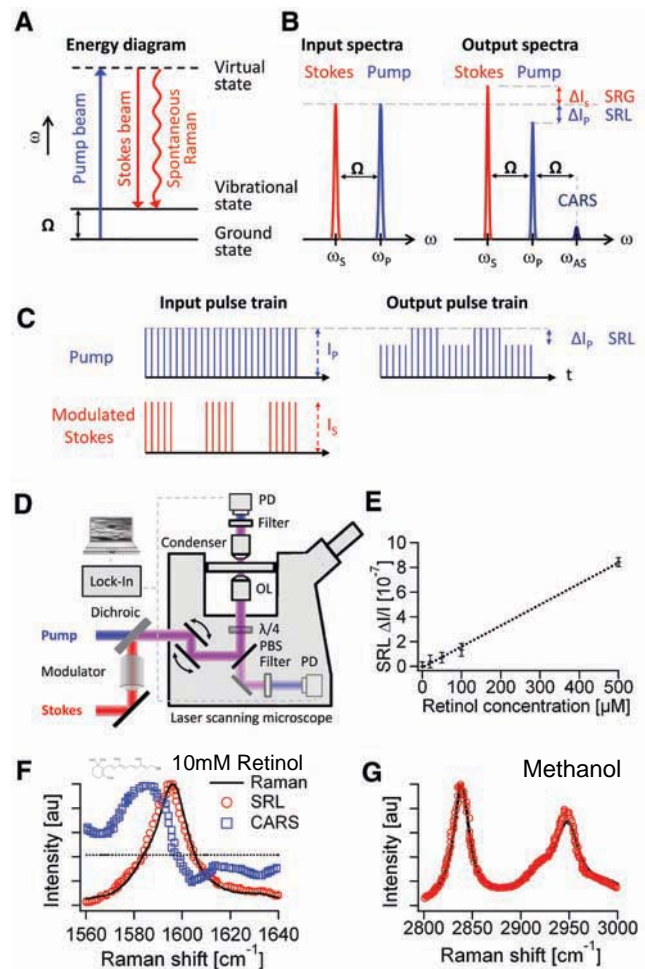
Alternatively, backward (epi) detection is possible in turbid samples because multiple scattering events redirect a considerable portion of the forward-propagating pump and Stokes beams to the backward direction, which can be collected with the same excitation objective lens spectra at a particular position be recorded by automated detected SRL instead of SRG sensitivity of the photodiode used.

Result verified that SRL is linear in both I_p and I_s . Unlike the CARS signal that is proportional to the square of the concentration, the linear dependence of SRL on analyte concentration (Fig. 1E) allows straightforward quantitative analysis. The detection limit is 50 μ M for retinol (Fig. 1E) and 5 mM for methanol

solutions (18), with average laser power <40 mW (30 MW/cm²) for each beam. Close to the shot noise limit, this sensitivity corresponds to about 3000 retinol and 300,000 methanol molecules in focus, respectively, which has surpassed the detection limit reported for CARS microscopy (23).

We show in Fig. 1F the SRL, spontaneous Raman, and CARS spectra of an isolated Raman peak of trans-retinol (18). Whereas SRL and spontaneous Raman spectra are nearly identical, the CARS spectrum exhibits a nonresonant background independent of the Raman shift, and spectral distortion because of interference with the background (24). Good agreement between the SRL, SRG, and spontaneous Raman spectra is also seen for spectra with multiple peaks (Fig. 1G) (18, 25). Thus, SRS allows simple spectroscopic identification based on the Raman literature, particularly in the “crowded” fingerprint region.

Fig. 1. Principle and design of SRS microscopy. (A) Energy diagram for SRS. (B) Input and output spectra of SRS. SRS leads to an intensity increase in the Stokes beam (SRG) and an intensity decrease in the pump beam (SRL). Also shown (not to scale) is the CARS signal generated at the anti-Stokes frequency ω_{AS} . (C) SRL detection scheme. Stokes beam is modulated at high frequency (MHz), at which the resulting amplitude modulation of the pump beam due to SRL can be detected. (D) SRL microscope with both forward and epi detection. The Stokes beam is modulated by an electro-optic modulator. The transmitted or reflected pump beam is filtered and detected by a large-area photodiode (PD). For epi detection, the back-scattered beams are collected by the excitation objective lens (OL) and separated from the excitation beams by a combination of a quarter wave plate ($\lambda/4$) and polarizing beam splitter (PBS). The SRL is measured by a lock-in amplifier to provide a pixel of the image. Three-dimensional images are obtained by raster-scanning the laser focus across the sample, and microspectroscopy can be performed by automated tuning of the pump wavelength. (E) Linear dependence of SRL on concentrations of retinol in ethanol at 1595 cm⁻¹. Modulation depth $\Delta I_p/I_p < 10^{-7}$ can be achieved. Error bars show 1 SD of the signals for a 1-min recording. The detection limit was determined to be 50 μ M. (F) Agreement of SRL spectrum (red circles) with the spontaneous Raman spectrum (black line) of the Raman peak (1595 cm⁻¹) of 10 mM retinol in ethanol. The distorted CARS spectrum (blue squares) exhibits a typical peak shift, dispersive shape and nonresonant background. (G) Agreement of the more complex SRL spectrum of methanol (red circles) with the spontaneous Raman spectrum (black line).



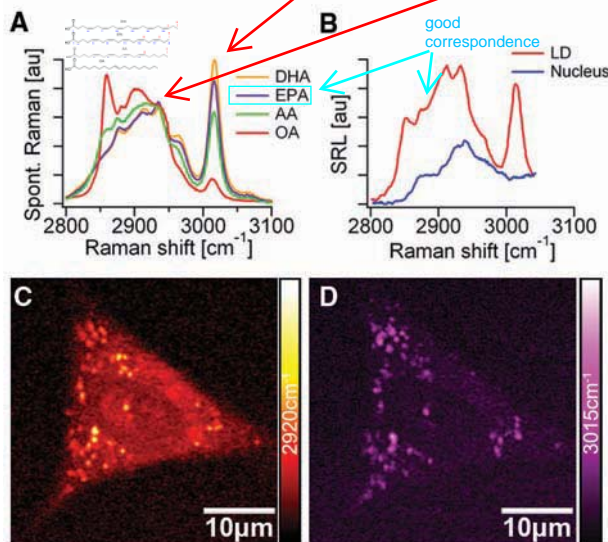
Imaging As the first application, we monitored the uptake of omega-3 fatty acids by living human lung cancer cells through SRL imaging and microspectroscopy (Fig. 2). Polyunsaturated omega-3 fatty acids, such as eicosapentaenoic acid (EPA), provide health benefits through mechanisms such as dampening inflammation, lowering blood tri-

glyceride concentrations, and inducing cancer cell apoptosis, but can only be obtained from the diet (26). As shown in Fig. 2A, unsaturated fatty acids exhibit a Raman band at 3015 cm^{-1} , attributable to the stretching mode of =C-H bond associated with C=C double bonds (27). The intensity of this 3015 cm^{-1} mode is approximately

proportional to the number of C=C double bonds in the lipid molecule. In contrast, the 2920 cm^{-1} peak intensity is found to be similar for all saturated and unsaturated fatty acids.

When cells are grown with 25 μM EPA for 24 hours (18), lipid droplets (LDs) are visible when imaging at both 2920 cm^{-1} (Fig. 2C) and 3015 cm^{-1} (Fig. 2D) bands. The SRL images show a much stronger signal outside the LDs at 2920 cm^{-1} than at 3015 cm^{-1} , indicating that most of the fatty acids outside the LDs are saturated. In the absence of EPA in the culturing media, the cells have few LDs inside the cytoplasm due to the limited lipid supply (18). We also conducted SRL microspectroscopy at specific positions inside the cell to identify the local chemical composition. The nucleus exhibits an SRL spectrum (blue in Fig. 2B) similar to that of the saturated fatty acids, with negligible contribution at 3015 cm^{-1} , whereas the spectrum from the LD has a pronounced 3015 cm^{-1} peak (red in Fig. 2B). No sign of photodamage, such as plasma membrane blebbing (15), was observed after repeated images of the same cell. Therefore, we can use SRL spectral imaging and microspectroscopy to follow uptake of unsaturated fatty acids by living cells, opening possibilities to study lipid metabolism and its associated diseases.

Fig. 2. Omega-3 fatty acid uptake by A549 human lung-cancer cells monitored with SRL microscopy and microspectroscopy. (A) Spontaneous Raman spectra of docosahexaenoic acid (DHA, with six C=C bonds), eicosapentaenoic acid (EPA, with five C=C bonds), arachidonic acid (AA, with four C=C bonds), and oleic acid (OA, with a single C=C bond). The strong Raman peak around 3015 cm^{-1} is characteristic of unsaturated fatty acids. (B) SRL spectra of a lipid droplet (LD, red line) and a region inside the nucleus (blue line). Unlike the nuclear region, the SRL spectrum of the LD shows good correspondence with the spectra from the pure EPA shown in (A). (C) SRL image of a cell at 2920 cm^{-1} . (D) SRL image of the same cell at 3015 cm^{-1} . These findings indicate that EPA is taken up by the cells and more strongly enriched in the LDs compared to other cellular organelles.



Next, we present SRS tissue imaging without staining. Many stains are impossible to apply in vivo. Label-free optical techniques, such as optical coherence tomography and diffusive optical tomography, often do not offer chemical contrast, while autofluorescence is limited to a few chemical species. A strong SRL signal originates from the CH_2 stretching vibration (2845 cm^{-1}) of lipids in tissue, especially in the brain, where lipid-rich myelin sheaths surround axons, as was seen in CARS microscopy (28). Figure 3A shows forward-detected SRL images of a fiber tract in the corpus callosum of a thin slice of mouse brain. We also demonstrate epi SRL imaging from a ~1-mm-thick slice of mouse brain (Fig. 3B), which clearly reveals individual neurons.

Skin imaging is another application of SRS microscopy. Figure 3C shows three individual SRL sections of mouse skin in the same area but at different depths, all with $\Delta\omega$ tuned into 2845 cm^{-1} (18). This highlights the 3D sectioning capability and subcellular resolution of SRS in tissue. At a depth of 4 μm , the SRL image shows the stratum corneum, which consists of polygonal cells and serves as the main protective layer of the body. This suggests that the intercellular space is rich in lipids. At a depth of 42 μm , lipid-rich sebaceous glands can be identified in the dermis. The nuclei of the gland cells are dark spots due to the lack of lipids. At a depth of 105 μm , the subcutaneous fat layer is clearly visible.

Figure 3D compares on and off vibrational resonance SRL and CARS images of stratum corneum. When $\Delta\omega$ is tuned from on-resonance (2845 cm^{-1}) to off-resonance (2780 cm^{-1}) of the

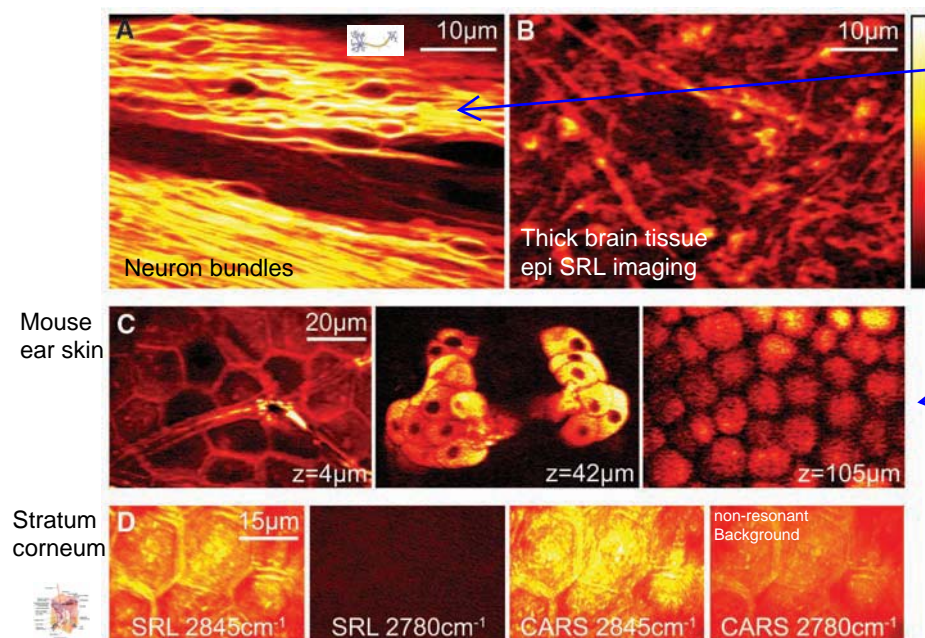


Fig. 3. SRL imaging of fresh mouse tissue. (A) Neuron bundles in corpus callosum of mouse brain imaged at 2845 cm^{-1} highlighting myelin sheaths rich in CH_2 . See movie in (18). (B) Epi-detected SRL CH_2 image acquired from thick brain tissue. (C) SRL CH_2 images of mouse ear skin in the same area at the indicated depths. From left to right: stratum corneum (4 μm), sebaceous gland (42 μm), and subcutaneous fat layer (105 μm). See movie in (18). (D) Comparison of SRL and CARS images of stratum corneum on (2845 cm^{-1}) and off (2780 cm^{-1}) the CH_2 resonance. Unlike CARS, SRL has no nonresonant background.

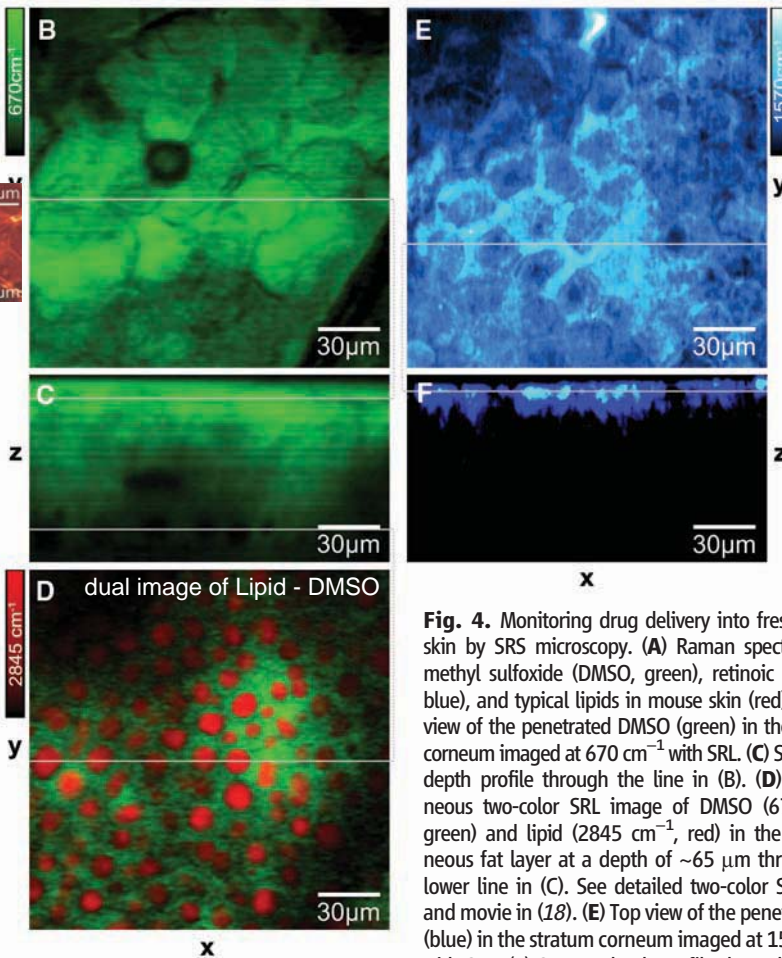
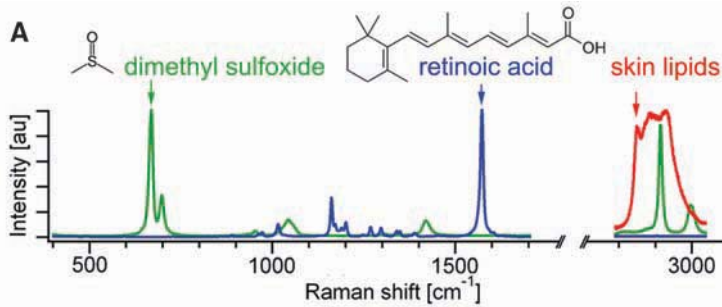


Fig. 4. Monitoring drug delivery into fresh mouse skin by SRS microscopy. (A) Raman spectra of dimethyl sulfoxide (DMSO, green), retinoic acid (RA, blue), and typical lipids in mouse skin (red). (B) Top view of the penetrated DMSO (green) in the stratum corneum imaged at 670 cm^{-1} with SRL. (C) SRL DMSO depth profile through the line in (B). (D) Simultaneous two-color SRL image of DMSO (670 cm^{-1} , green) and lipid (2845 cm^{-1} , red) in the subcutaneous fat layer at a depth of $\sim 65 \mu\text{m}$ through the lower line in (C). See detailed two-color SRL setup and movie in (18). (E) Top view of the penetrated RA (blue) in the stratum corneum imaged at 1570 cm^{-1} with SRL. (F) SRL RA depth profile through the line

in (E). SRS allows label-free 3D in situ visualization of two different drug-delivery pathways into the skin.

CH_2 stretching mode, the SRL signal vanishes completely, whereas the nonresonant CARS background still exhibits contrast that complicates image interpretation. We note that tissue autofluorescence does not interfere with the SRS. The absence of the nonresonant background in SRS reflects the major advantage over CARS imaging.

We also show the use of SRS to monitor drug delivery. Fluorescent labels are usually larger than drug molecules and may perturb their transport properties. Although confocal spontaneous Raman microspectroscopy has been used to obtain longitudinal penetration profiles, the lateral distribution is often compromised due to the long pixel dwell times (29). Here we show the mapping of the distribution of two com-

pounds: dimethyl sulfoxide (DMSO), a skin-penetration enhancer (29); and retinoic acid (RA), which is used to treat acne, wrinkles, photoaging, and acute promyelocytic leukemia (30). According to Raman spectra (Fig. 4A), DMSO and RA have isolated vibrational resonances at 670 and 1570 cm^{-1} , respectively.

As a hydrophilic molecule, DMSO penetrates the skin via the protein phase, so the DMSO image in the stratum corneum (Fig. 4B) shows inverse contrast compared to the lipid image in Fig. 3C. A depth profile shows detectable DMSO over more than 60 μm (Fig. 4C), and the hydrophilic interaction with the tissue is confirmed in the subcutaneous fat layer. Simultaneous two-color imaging tuned into lipid and DMSO (18)

allows us to show that the DMSO is insoluble in the lipid structures (Fig. 4D). In contrast, RA, which is a hydrophobic molecule, penetrates via the lipid-rich intercellular space in the epidermis (Fig. 4, E and F) after ultrasonication of the tissue to enhance delivery (18). These results show that SRS offers a new approach for studying pharmacokinetics in situ. As a label-free and sensitive imaging modality, SRS microscopy allows mapping of molecular species in 3D and the ability to follow their dynamics in living cells and organisms based on the wealth of Raman spectroscopy.

References and Notes

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Global Medical. Harvard University has filed a patent application based on this work.

Supporting Online Material

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Materials and Methods

Figs. S1 to S6
Movies S1 to S3

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