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Highly Sensitive Vibrational Imaging by Femtosecond Pulse Stimulated Raman Loss

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Abstract

Nonlinear vibrational imaging of live cells and organisms is demonstrated by detecting femtosecond pulse stimulated Raman loss. Femtosecond pulse excitation produced a 12 times larger stimulated Raman loss signal than picosecond pulse excitation. The large signal allowed real-time imaging of the conversion of deuterated palmitic acid into lipid droplets inside live cells, and three-dimensional sectioning of fat storage in live *C. elegans*. With the majority of the excitation power contributed by the Stokes beam in the 1.0 to 1.2 μm wavelength range, photodamage of biological samples was not observed.

Keywords

stimulated Raman scattering (SRS); nonlinear microscopy; quantitative analysis; 3D imaging; femtosecond laser; coherent Raman scattering; lipid storage

Label-free vibrational imaging is an attractive alternative to fluorescence imaging. Nonlinear vibrational microscopy based on coherent anti-Stokes Raman scattering (CARS) has found broad applications [1-3], especially in the study of lipid bodies and white matter based on the strong resonant signal from C-H stretch vibration [4]. Various methods, including polarization-sensitive detection [5], time-delayed detection [6], frequency modulation [7, 8], and phase-sensitive heterodyne detection [9, 10], have been developed for suppression of the nonresonant background. Despite appealing proof-of-principle demonstrations, the complexity of these advanced methods hinders their biological applications. Stimulated Raman scattering (SRS) microscopy provides a straightforward way to solve the nonresonant background problem [11-14]. The SRS process occurs simultaneously with CARS, represented by an intensity gain in the Stokes field and an intensity loss in the pump field. Unlike the CARS process where the signal is produced at a new frequency, the signal in SRS is generated at the frequency of incident beams which provide a local oscillator E_{LO} to mix with the signal field E_{sig} . Because only the vibrationally resonant signal is mixed with the local oscillator, such heterodyne detection removes the nonresonant background. Additionally, mixing with the intense local oscillator boosts the signal level. The SRS signal provides spectral information identical to spontaneous Raman and is linearly proportional to the molecular concentration. These advantages facilitate data interpretation and also enhance the detection sensitivity for low-concentration molecules.

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#Equal contributions

Supporting Information **Available:** Spatial resolution of fs SRL microscope (Fig S1), SRL spectrum of PLGA film (Fig. S2), SRL movies (Movie S1-S2), including depth sectioning, 3D rendering of *C. elegans* at C-H vibration, and 3D rendering of simultaneous TPEF imaging (Movie S3). This material is available free of charge via the Internet <http://pubs.acs.org>.

SRS imaging was first demonstrated on polymer beads using amplified femtosecond (fs) pulses of low repetition rate [11]. By making use of MHz frequency modulation to reject the low-frequency laser noise, SRS imaging on the order of a few tens of seconds per image of 512×512 pixels has been demonstrated [12, 13]. Very recently, video-rate SRS imaging has been realized by using a fast lock-in amplifier [15]. Technically SRS imaging can be implemented on a CARS microscope by adding a function generator, an optical modulator, a lock-in amplifier and a photodiode detector. SRS microscopy has been applied to vibrational imaging of tablets and biomass based on fingerprint Raman bands [16, 17].

In spite of these advances, the detection sensitivity of SRS imaging can be much improved. So far high-speed SRS imaging has been implemented with picosecond (ps) pulse excitation, where the signal level is limited by the relatively low peak power of ps pulses. Because shot noise limit has been reached with mode-locked lasers [12], an effective way to improve the detection limit is to increase the SRS signal level by shorter pulse excitation. Historically laser pulses of a few ps in duration were promoted for CARS imaging in order to increase the ratio of resonant signal to non-resonant background [18]. However, such requirement is eliminated in SRS microscopy because the SRS signal is free of the non-resonant background. Femtosecond pulse excitation has been used for background-free SRS spectroscopy [19, 20]. In this paper we show theoretically and experimentally that, by using fs pulse excitation, the signal to noise ratio can be increased by one order of magnitude for high-speed, bond-selective imaging of isolated Raman bands, including the C-H and C-D stretch vibrations.

We adopted a frequency domain model [18] to evaluate the relationship between the coherent Raman scattering intensity and pulse width. In the frequency domain, the stimulated Raman loss (SRL) field arises from the induced third-order polarization written as

$$P^{(3)}(\omega_{sig}) = \int_{-\infty}^{+\infty} d\omega_s \int_{-\infty}^{+\infty} d\omega'_s \int_{-\infty}^{+\infty} d\omega_p \chi_{SRL}^{(3)} E_s(\omega_s) E_s(\omega'_s) E_p(\omega_p) \delta(\omega_s - \omega'_s + \omega_p - \omega_{sig}), \quad (1)$$

where $E_s(\omega_s)$ and $E_p(\omega_p)$ denotes the Stokes and pump fields, respectively. The third-order susceptibility is written as

$$\chi_{SRL}^{(3)} = \chi_{NR}^{(3)} + \frac{A}{\omega_s - (\omega_p - \Omega) + i\Gamma}. \quad (2)$$

Here $\chi_{NR}^{(3)}$ is the nonresonant contribution. The second term is the vibrationally resonant part $\chi_R^{(3)}$, where A is a constant. The pump and Stokes fields are assumed to be two temporally overlapped pulses with Gaussian spectral profiles,

$$\begin{aligned} E_p(\omega_p) &= \frac{A_p}{\Delta_p^{1/2}} \exp \left\{ \frac{-2(\omega_p^0 - \omega_p)^2 \ln 2}{\Delta_p^2} \right\}, \\ E_s(\omega_s) &= \frac{A_s}{\Delta_s^{1/2}} \exp \left\{ \frac{-2(\omega_s^0 - \omega_s)^2 \ln 2}{\Delta_s^2} \right\}. \end{aligned} \quad (3)$$

Here ω_p^0 and ω_s^0 are central frequencies of the pump and Stokes fields. Δ_p and Δ_s are the spectral full widths at half maximum (FWHM) of the pump and Stokes fields, respectively. A_p and A_s are constants related to peak intensities. To simplify the calculation, we assume $\Delta_p = \Delta_s$. The prefactors in Eq. 3 ensure that the pulse energy is independent of the pulse spectral width. The heterodyne-detected SRL intensity at ω_{sig} is written as

$$\begin{aligned} \Delta I_p(\omega_{sig}) &= 2 \int_{-\infty}^{+\infty} d\omega_s \int_{-\infty}^{+\infty} d\omega'_s \int_{-\infty}^{+\infty} d\omega_p \int_{-\infty}^{+\infty} d\omega'_p \text{Im}(\chi_{SRL}^{(3)}) E_s(\omega_s) E_s(\omega'_s) E_p(\omega_p) E_p(\omega'_p) \cdot \delta(\omega_s - \omega'_s + \omega_p - \omega_{sig}) \delta(\omega_{sig} - \omega'_p) \end{aligned} \quad (4)$$

Here the second delta function represents the requirement for mixing between the local oscillator and the SRL field.

Based on the above theoretical model we have computed the relationship between the pulse spectral width and the spectrally integrated SRL intensity,

$$I_{SRL} = - \int_{-\infty}^{\infty} \Delta I_p(\omega_{sig}) d\omega_{sig}. \quad (5)$$

Our calculation (Fig. 1) indicates that $\Delta I_p(\omega_{sig}=\omega_p^0)$ strongly depends on the Raman line half-width and the pulse spectral width. For $\Gamma = 5 \text{ cm}^{-1}$, ΔI_p is amplified by 6 times when the spectral width increases from 1 cm^{-1} to 20 cm^{-1} and becomes saturated thereafter. However, for $\Gamma = 25 \text{ cm}^{-1}$, ΔI_p is amplified by 30 times when the pulse spectral width increases from 1 cm^{-1} ($\sim 14 \text{ ps}$) to 120 cm^{-1} ($\sim 120 \text{ fs}$). The same results hold for stimulated Raman gain (SRG). Therefore, by use of shorter pulses, we expect to increase the SRL signal level by one order of magnitude at the same excitation energy.

A practical challenge of using fs pulse excitation is the increased photodamage potential associated with the high peak power of fs pulses. To minimize the multiphoton absorption-induced photodamage, we adopted an SRL configuration in which most excitation power was carried by the Stokes beam in the 1.0 to $1.2 \mu\text{m}$ wavelength range. The SRL setup is shown in Fig. 2. A Ti:Sapphire laser (Chameleon Vision, Coherent) with up to 4W (80MHz , $\sim 140 \text{ fs}$ pulse width) pumps an optical parametric oscillator (OPO, Chameleon Compact, Angewandte Physik & Elektronik GmbH), providing the pump beam tunable from 680 to 1080 nm and the Stokes beam tunable from 1.0 to $1.6 \mu\text{m}$. The pump and Stokes pulse trains were collinearly overlapped and directed into a laser-scanning microscope (FV300, Olympus). A $60\times$ water-immersion objective lens (UPlanSApo, Olympus) was used to focus the laser into a sample. To minimize the thermal lensing effect, another water-immersion objective lens (LUMFI, Olympus) of 1.10 numerical aperture (NA) was used to collect the signal in a forward direction. The Stokes beam intensity was modulated by an acousto-optic modulator (15180-1.06-LTD-GAP, Gooch & Housego) with 70% modulation depth. The shot noise limit was reached by modulating the Stokes beam intensity at 5.4 MHz (Fig. 2). The SRL signals were detected by a photodiode (818-BB-40, Newport) and then sent to a fast lock-in amplifier (HF2LI, Zurich Instrument) which has a time constant as small as 800

ns. The lateral and axial resolutions of our SRS microscope are about 0.42 and 1.01 μm , respectively, measured from the X-Z intensity profile of 200-nm polystyrene beads (Fig. S1).

To confirm the theoretical results, we compared the SRL intensity from C-H stretching of olive oil generated by 5-ps and 200-fs laser pulses, respectively. The 5-ps laser system for SRS imaging was described in [16]. We used 6 mW for the pump and 6 mW for the Stokes at the sample. The intensity profiles below the SRL images (Fig. 3) show that the signal level increased more than 12 times when the excitation was switched from 5-ps to 200-fs pulsed lasers. Meanwhile, the noise level remained the same.

Using the fs laser based SRL microscope, we explored the uptake of palmitic acid and its intracellular fate in Chinese hamster ovary (CHO) cells. Excess palmitic acids have been shown to induce lipo-toxicity in mammalian cells [21]. However, direct visualization of the fatty acids was not accessible with existing imaging tools. To selectively detect the molecule by SRL, we used deuterated palmitic acid- d_{31} (Aldrich) which gives an isolated Raman band at 2110 cm^{-1} corresponding to the C-D bond stretch vibration (Fig. 4A). The deuterated compound was clearly visualized in lipid droplets (LDs) and also in the cellular membranes (Fig. 4C, 4E). The C-D signal from LDs composed $19 \pm 5\%$ of total intensity in the group treated with palmitic acid alone, whereas the LD signal composed $32 \pm 4\%$ of total intensity in the group treated by both palmitic acid and methyl oleate. These results provide visual evidence that oleic acid facilitates the conversion of palmitic acid into lipid bodies [21]. Previously picosecond SRS was used to image cellular uptake of fatty acids but only the LDs were detected [12]. The increased sensitivity by fs pulse excitation allowed us to detect the fatty acids in cell membranes. We also imaged the same cells based on the SRL signal from C-H vibration (Fig. 4F-I). It was found that the C-D rich droplets were overlapped with the C-H abundant droplets. In the control cells and the oleate-treated cells, the SRL signals at the C-D vibration frequency was not detected (Fig. 4B, 4D), which confirmed the bond-selective imaging capability of the SRL setup.

As another important application, we demonstrated SRL imaging of fat storage in live *Caenorhabditis elegans* (*C. elegans*). As a label-free imaging modality, CARS microscopy has been employed to selectively visualize LDs in *C. elegans* [22, 23]. More recently, SRS microscopy has been used to map lipids in the worm but without 3D sectioning [24]. By using an objective lens of 1.2 NA, we obtained 3D SRL images of a wild type *C. elegans* using the signal from C-H bond (Fig. 5A, Movie S1-S2). Such 3D sectioning is critical to distinguish the fat stored in LDs from the membrane lipids. As an additional advantage, our system with fs pulse excitation is highly compatible with other modalities such as two photon excitation fluorescence (TPEF). This advantage allows simultaneous forward SRL imaging of lipid and backward TPEF imaging of autofluorescence in *C. elegans*. As shown in Fig. 5B and Movie S3, the autofluorescence arose from the small intestine and some particles that were not overlapped with the LDs.

In our setup, most excitation power is carried by the Stokes beam at a wavelength above 1.0 μm , with the pump beam power being as low as a few mW at the sample. Thus photodamage to cells is not expected. To explore the phototoxicity, we determined the power threshold of cell damage at different wavelengths (Fig. 6). We used plasma membrane blebbing [25] as an indicator of cell damage. At the wavelengths of 680, 830, 880, and 1000 nm, the power density at the sample to cause CHO cell membrane blebbing was found to be 1.6, 8.9, 10.5, and 15.8 MW/cm^2 , respectively. At 1100 nm, we did not observe photodamage with the maximum power (9.5 MW/cm^2 at sample) provided by the OPO. In our SRL imaging experiment, the maximum power density used was 2.9 MW/cm^2 for 830 nm, 5.8 MW/cm^2 for 1000 nm and 7.8 MW/cm^2 for 1100 nm, which was far below

the damage threshold. Accordingly, no damage was observed during the imaging procedures.

Although the resolution of fs pulses is known to be insufficient for studying the fingerprint region, the spectral resolution of our setup is sufficient for imaging isolated Raman bands such as the C-D stretch band at 2100 cm^{-1} . We examined the spectral resolution of our setup using the C-H stretch bands of poly(lactic-co-glycolic acid) (PLGA). The Lorentzian fit of the fs SRL spectrum of the 2960 cm^{-1} band of PLGA (Fig. S2) produced a FWHM about 100 cm^{-1} . Such value is smaller than the calculated FWHM (136 cm^{-1}) of SRL spectrum given by Eq. 4 based on the pulse widths of the pump field (140 fs), the Stokes field (200 fs), and the FWHM of the Raman line (26 cm^{-1}). The enhanced spectral resolution is possibly due to the pulse chirping caused by the optics inside the microscope. Spectral focusing of fs pulses can be achieved by introduction of chirping in the optical path [26, 27].

To explore the effect of pulse broadening on the SRL signal level, we measure the SRL signal from oil as a function of temporal overlap of the two pulsed beams and obtained a FWHM of ~ 480 fs. This result indicates some broadening of the laser pulses at sample. Nevertheless, such broadening does not reduce the SRL signal according to Fig. 1. Theoretically, transform-limited 1-ps pulses are optimal for coherent Raman imaging. Such pulses are however difficult to produce in solid state lasers. In this work, the fs pulses not only produce a strong SRS signal, but also facilitate the coupling of SRS with other NLO modalities such as two-photon fluorescence and second harmonic generation. Our study shows that SRS imaging can be implemented on a widely used multiphoton microscope platform. Importantly, the photodamage is negligible in the SRL configuration where most excitation power is carried by the Stokes beam in the 1.0 to $1.2\text{ }\mu\text{m}$ region. These results are expected to expedite the biological applications of SRS microscopy.

To summarize, we have shown a new platform for real-time vibrational imaging. Compared to existing SRS microscopes with ps laser excitation, fs excitation increases the signal intensity by one order of magnitude. We have compared the new setup with an SRS system pumped with 5-ps laser at the same power and found a 12-fold increase in signal to noise ratio for imaging C-H bonds. Though we focused C-D and C-H bonds, the broad tuning range of the OPO (from 1.0 to $1.6\text{ }\mu\text{m}$) also permit SRL imaging of O-D, S-H, N-H, and O-H bonds based on their stretching vibrations. Because the majority of the excitation power is carried by the Stokes beam above $1.0\text{ }\mu\text{m}$ in wavelength, photodamage to the sample is negligible. As an additional advantage, the fs pulse based SRL modality is highly compatible with other NLO modalities including TPEF, which facilitates multimodal imaging of complex biological tissues. These advances open up new opportunities for label-free molecular imaging of live cells and organisms using signals from molecular vibration.

Experimental Section

Live cell imaging

Deuterated palmitic acid was dissolved in DMSO and added to the cell culture medium at a final concentration of $100\text{ }\mu\text{M}$. After 7 hours of incubation, the CHO cells were directly imaged with the SRL microscope. To study the conversion of the deuterated palmitic acid, 10 sectioning of CHO cells of each group were used for analysis.

Live *C. elegans* imaging

Wild type *C. elegans* were transferred from a petri plate to a thin layer of agar gel with a home-made worm-picker. The worms were placed on top of the agar gel and anesthetized by 100 mM sodium azide solution. The sample in $\sim 340\text{ }\mu\text{m}$ thick agar gel was sandwiched between two coverslips and was imaged immediately.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Müller M, Zumbusch A. Coherent anti-Stokes Raman scattering microscopy. *ChemPhysChem*. 2007; 8:2156–2170. [PubMed: 17768730]
2. Cheng JX. Coherent anti-Stokes Raman scattering microscopy. *Appl Spectrosc*. 2007; 61(9):197A–206A. [PubMed: 17910784]
3. Evans CL, Xie XS. Coherent anti-Stokes Raman scattering microscopy: chemically selective imaging for biology and medicine. *Annu Rev Anal Chem*. 2008; 1:883–909.
4. Le TT, Yue S, Cheng JX. Shedding new light on lipid biology by CARS microscopy. *J Lipid Res*. 2010; 51:3091–3102. [PubMed: 20713649]
5. Cheng JX, Book LD, Xie XS. Polarization coherent anti-Stokes Raman scattering microscopy. *Opt Lett*. 2001; 26:1341–1343. [PubMed: 18049602]
6. Volkmer A, Book LD, Xie XS. Time-resolved coherent anti-Stokes Raman scattering microscopy: Imaging based on Raman free induction decay. *Appl Phys Lett*. 2002; 80:1505–1507.
7. Ganikhanov F, Evans CL, Saar BG, Xie XS. High-sensitivity vibrational imaging with frequency modulation coherent anti-Stokes Raman scattering (FM-CARS) microscopy. *Opt Lett*. 2006; 31:1872–1874. [PubMed: 16729099]
8. Saar BG, Holtom GR, Freudiger CW, Ackermann C, Hill W, Xie XS. Intracavity wavelength modulation of an optical parametric oscillator for coherent Raman microscopy. *Opt Express*. 2009; 17:12532–12539. [PubMed: 19654655]
9. Potma EO, Evans CL, Xie XS. Heterodyne coherent anti-Stokes Raman scattering (CARS) imaging. *Opt Lett*. 2006; 31(2):241–243. [PubMed: 16441043]
10. Jurna M, Kortner JP, Otto C, Herek JL, Offerhaus HL. Background free CARS imaging by phase sensitive heterodyne CARS. *Opt Express*. 2008; 16:15863–15869. [PubMed: 18825222]
11. Ploetz E, Laimgruber S, Berner S, Zinth W, Gilch P. Femtosecond stimulated Raman microscopy. *Appl Phys B*. 2007; 87:389–393.
12. Freudiger CW, Min W, Saar BG, Lu S, Holtom GR, He C, Tsai JC, Kang JX, Xie XS. Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy. *Science*. 2008; 322(5909):1857–1861. [PubMed: 19095943]
13. Nandakumar P, Kovalev A, Volkmer A. Vibrational imaging based on stimulated Raman scattering microscopy. *New J Phys*. 2009; 11:033026.
14. Ozeki Y, Dake F, Kajiyama S, Fukui K, Itoh K. Analysis and experimental assessment of the sensitivity of stimulated Raman scattering microscopy. *Optics Express*. 2009; 17(5):3651–3658. [PubMed: 19259205]
15. Saar BG, Freudiger CW, Reichman J, Stanley CM, Holtom GR, Xie XS. Video-rate molecular imaging in vivo with stimulated Raman scattering. *Science*. 2010; 330(6009):1368. [PubMed: 21127249]
16. Slipchenko MN, Chen H, Ely DR, Jung Y, Carvajal MT, Cheng JX. High-speed and high-resolution vibrational imaging of tablets by epi-detected stimulated Raman scattering microscopy. *Analyst*. 2010; 135:2613–2619. [PubMed: 20625604]
17. Saar BG, Zeng Y, Freudiger CW, Liu YS, Himmel ME, Xie XS, Ding SY. Label-Free, Real-Time Monitoring of Biomass Processing with Stimulated Raman Scattering Microscopy. *Angew Chem Int Ed*. 2010; 49:5476–5479.

18. Cheng JX, Volkmer A, Book LD, Xie XS. An epi-detected coherent anti-Stokes Raman scattering (E-CARS) microscope with high spectral resolution and high sensitivity. *J Phys Chem B*. 2001; 105:1277–1280.
19. McCamant DW, Kukura P, Mathies RA. Femtosecond broadband stimulated Raman: A new approach for high-performance vibrational spectroscopy. *Appl Spectrosc*. 2003; 57(11):1317–1323. [PubMed: 14658143]
20. Kukura P, McCamant DW, Mathies RA. Femtosecond stimulated Raman spectroscopy. *Annu Rev Phys Chem*. 2007; 58:461–488. [PubMed: 17105414]
21. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A*. 2003; 100(6):3077. [PubMed: 12629214]
22. Le TT, Duren HM, Slipchenko MN, Hu CD, Cheng JX. Label-free quantitative analysis of lipid metabolism in living *Caenorhabditis elegans*. *J Lipid Res*. 2010; 51(3):672. [PubMed: 19776402]
23. Yen K, Le TT, Bansal A, Narasimhan SD, Cheng JX, Tissenbaum HA, Melov S. A comparative study of fat storage quantitation in nematode *Caenorhabditis elegans* using label and label-free methods. *PloS one*. 2010; 5(9):387–398.
24. Wang MC, Min W, Freudiger CW, Ruvkun G, Xie XS. RNAi screening for fat regulatory genes with SRS microscopy. *Nat Methods*. 2011
25. Fu Y, Wang H, Shi R, Cheng JX. Characterization of photodamage in coherent anti-Stokes Raman scattering microscopy. *Opt Express*. 2006; 14(9):3942–3951. [PubMed: 19516542]
26. Hellerer T, Enejder AMK, Zumbusch A. Spectral focusing: High spectral resolution spectroscopy with broad-bandwidth laser pulses. *Appl Phys Lett*. 2004; 85(1):25–27.
27. Pegoraro AF, Ridsdale A, Moffatt DJ, Jia Y, Pezacki JP, Stolow A. Optimally chirped multimodal CARS microscopy based on a single Ti:sapphire oscillator. *Opt Express*. 2009; 17:2984–2996. [PubMed: 19219203]

Figure 1. Calculated SRS intensities for narrow $\Gamma = 5 \text{ cm}^{-1}$ and broad $\Gamma = 25 \text{ cm}^{-1}$ Raman transitions

The intensities are normalized by the values at the pulse spectral width of 1.0 cm^{-1} . Γ is the Raman line half-width.

Figure 2. The femtosecond pulse stimulated Raman loss imaging system

The top left box is the electronic connection, where solid line indicates electronic control and dashed line as data flow. Inset shows the shot noise limit detection at megahertz modulation. The PD and PMT are used for forward SRL and backward two photon fluorescence detection, respectively. LIA: lock-in amplifier. AOM: acousto-optic modulator. PD: photodiode. SU: Scanning unit. DM: dichroic mirror. PMT: photomultiplier tube.

Figure 3. Experimental comparison of SRL signal levels between picosecond and femtosecond excitation

(A) SRL image of C-H stretching vibration at the interface of olive oil and air, with 5 ps excitation. (B) SRL image of the same sample with 200-fs pulse excitation. The same power of 6 mW ($1.1 \text{ MW}/\text{cm}^2$) at the sample was used for both pump and Stokes fields. Shown below each image is the intensity profile along the dashed line. Bar: 20 μm .

Figure 4. SRL imaging of deuterated lipids in live CHO cells

(A) Molecular structure and Raman spectrum of palmitic acid-d₃₁. (B-I) SRL images of C-D stretch vibration and C-H stretch vibration. (J-M) Transmission images in accordance with each column. Column one to four: control, deuterated palmitic acid alone, oleate alone, and deuterated palmitic acid + oleate. The cells were treated for 7 hours. All images were acquired at the speed of 4 μ s per pixel. The pump and Stokes power at the sample was 10 mW and 40 mW, respectively. The pump wavelength was 830 nm. The Stokes wavelength was 1090 nm for C-H and 1005 nm for C-D. Bar: 5 μ m.

Figure 5. SRL imaging of fat storage in *C. elegans*

Depth-resolved SRL images of a live *C. elegans* based on the signal from C-H stretching band (A) and simultaneous TPEF images of autofluorescence (B). A total of 50 frames were taken at the axial step of 1.0 μm . The images of 512 by 512 pixels were acquired at the speed of 2 μs per pixel. The same power as in Figure 4 was used. Bar: 20 μm . Videos of the 3D rendering are available in supplementary materials (Movie S1-S2).

Figure 6. Characterization of photodamage as a function of fs laser wavelength

(A) Transmission image of non-damaged cells. (B) Damaged cells indicated by blebblings of the plasma membranes (arrows). Bar: 10 μm . (C) Damage threshold at different laser wavelengths. In all experiments, CHO cells were continuously scanned for 2 min.