

Journal of Visualized Experiments

Label-Free Imaging of Lipid Storage Dynamics in Caenorhabditis elegans using Stimulated Raman Scattering Microscopy

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61870R1
Full Title:	Label-Free Imaging of Lipid Storage Dynamics in Caenorhabditis elegans using Stimulated Raman Scattering Microscopy
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Additional Information:	
Question	Response
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TITLE:

Label-Free Imaging of Lipid Storage Dynamics in *Caenorhabditis elegans* using Stimulated Raman Scattering Microscopy

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KEYWORDS:

Raman imaging; Stimulated Raman Scattering microscopy; SRS microscopy; *C. elegans*; lipid metabolism; lipids; fat; lipid droplets; label-free imaging; lipid imaging; live imaging

SUMMARY:

Stimulated Raman scattering (SRS) microscopy allows selective, label-free imaging of specific chemical moieties and it has been effectively employed to image lipid molecules in vivo. Here, we provide a brief introduction to the principle of SRS microscopy and describe methods for its use in imaging lipid storage in *Caenorhabditis elegans*.

ABSTRACT:

Lipid metabolism is a fundamental physiological process necessary for cellular and organism health. Dysregulation of lipid metabolism often elicits obesity and many associated diseases including cardiovascular disorders, type II diabetes, and cancer. To advance the current understanding of lipid metabolic regulation, quantitative methods to precisely measure in vivo lipid storage levels in time and space have become increasingly important and useful. Traditional approaches to analyze lipid storage are semi-quantitative for microscopic assessment or lacking spatio-temporal information for biochemical measurement. Stimulated Raman scattering (SRS) microscopy is a label-free chemical imaging technology that enables rapid and quantitative detection of lipids in live cells with a subcellular resolution. As the contrast is exploited from intrinsic molecular vibrations, SRS microscopy also permits four-dimensional tracking of lipids in live animals. In the last decade, SRS microscopy has been widely used for small molecule imaging in biomedical research and overcome the major limitations of conventional fluorescent staining and lipid extraction methods. In the laboratory, we have combined SRS microscopy with the

genetic and biochemical tools available to the powerful model organism, *Caenorhabditis elegans*, to investigate the distribution and heterogeneity of lipid droplets across different cells and tissues and ultimately to discover novel conserved signaling pathways that modulate lipid metabolism. Here, we present the working principles and the detailed setup of the SRS microscope and provide methods for its use in quantifying lipid storage at distinct developmental timepoints of wild-type and insulin signaling deficient mutant *C. elegans*.

INTRODUCTION:

Obesity has become a global health problem threatening one third of the population around the world, and it presents a serious medical concern, given its association with poor mental health¹ and deadly diseases including diabetes², cardiovascular diseases³ and some types of cancer⁴. Study of lipid metabolism is essential to better understand the biological problems behind obesity. Rapid and specific quantification of lipid storage entails the detection of fatty acids and their derivatives, as well as sterol-containing metabolites, with high sensitivity and preferably with spatial information. Lipids are challenging targets to image because they lack intrinsic fluorescence and cannot be easily fluorescently tagged. The fluorescent tags are often larger than the lipid molecules and, therefore, can be chemically invasive and impractical for in vivo applications. Label-free or minimal labeling strategy is necessary to preserve the hydrophobic structure of the lipid molecules⁵. Recent developments in imaging technologies have created exciting opportunities for label-free imaging of lipids in living cells, tissues, and organisms.

Traditional approaches for lipid storage analyses in biological samples include biochemical assays and staining protocols with lipophilic dyes. Biochemical quantification assays involving mass spectrometry (MS) are unmatched in their molecular resolvability, but they require very large sample amounts and sample preparation usually takes several hours, limiting their application for real-time imaging of living systems⁵. Another major limitation of these assays is the lack of spatial information. On the other hand, lipophilic dyes such as Oil Red O and Sudan Black provide tissue and cellular distribution of lipid storage organelles and compared to MS techniques, these staining methods are also low in cost and easy to perform. However, these staining protocols require fixation, which can impact the hydrophobic nature of the lipid droplets, generate artificial changes in their structure and result in inconsistencies between experiments⁶. The technical difficulties associated with biochemical and staining techniques have led to the search for label-free methods to image lipid molecules and to the rapid increase in the usage of coherent Raman scattering (CRS) microscopy in lipid imaging.

The Raman effect was first recognized by Raman and Krishnan, where they reported that upon interacting with a photon, a molecule can generate scattered light with no change in wavelength (called Rayleigh scattering) or rarely with an altered wavelength (called Raman scattering) and this change in wavelength is characteristic of the functional chemical groups within the molecule⁷. When the chemical bonds within a molecule get excited to a higher vibrational energy level by an incident photon, called pump photon, the energy of the scattered photon, called the Stokes photon, become lower. Otherwise, the chemical bonds can reach a lower vibrational energy level if they are originally at a higher level, and the scattered photon gain energy to be the anti-Stokes photon. The frequency difference between the incident and scattered photons is

known as the Raman shift. Each chemical bond within a molecule has a characteristic and quantifiable Raman shift. For example, the CH₂ bond has a Raman shift of 2,845 cm⁻¹, which is abundant in fatty acid chains⁸. This spontaneous Raman signal is generally very weak, which has vastly limited the imaging speed in conventional spontaneous Raman microscopy. Over the years, various approaches have been developed to increase the imaging speed and sensitivity of spontaneous Raman microscopy. Coherent Raman scattering microscopy, including Coherent Anti-Stokes Raman Scattering (CARS) microscopy and Stimulated Raman Scattering (SRS) microscopy, is the most recent progress. CARS and SRS have slightly different working principles, but both are label-free techniques that have live imaging capability, can yield spatial and temporal information on lipid storage dynamics, and only require small sample size. CARS microscopy suffers from non-resonant background, which comes from various non-linear processes, and CARS signals also have non-linear relationship with the molecule concentration, which together complicate the quantification process⁹. Unlike CARS microscopy, SRS microscopy does not generate non-resonant background signals and offers linear dependence on the concentration of the molecule of interest. Thus, currently SRS microscopy is more widely used for lipid imaging.

In SRS microscopy, the weak spontaneous Raman signals can be amplified when excited by two synchronized laser beams with their frequency difference matching the chemical bond vibrational frequency. The molecule will experience an enhanced transition to an excited state due to coherent excitation. As a result, the rate of Stokes photon generation is boosted. Consequently, the intensity of the transmitted “Stokes” beam increases (stimulated Raman gain, SRG) and the intensity of the transmitted “pump” beam decreases (stimulated Raman loss, SRL). Detection of SRG or SRL signals underlies the basis for Stimulated Raman Scattering (SRS) microscopy imaging of molecules with specific chemical bonds¹⁰. If the frequency difference between the two laser beams does not match the vibrational frequency of the chemical bond within a molecule of interest, neither SRG nor SRL signals will be generated. The imaging speed of SRS microscopy is around 2 μsec per pixel or 1 second per frame, which is much faster than spontaneous Raman microscopy¹¹. Typical lateral resolution for SRS microscopy is diffraction limited and around 300 nm. In addition, the two-photon optical processes of SRS microscopy allow for volumetric 3D imaging of relative thick tissue samples and the imaging depth could reach 300–500 μm. Overall, SRS microscopy presents an efficient, label-free imaging technique to detect specific biomolecules, especially lipids.

Lipid droplets are single-membrane organelles, which are the main cellular storage site for neutral lipids, including the triacylglycerols (TAGs) and cholesterol esters (CEs). CH₂ bonds in the fatty acid chains of these lipid molecules generate strong SRS signals at 2,845 cm⁻¹ when excited⁸, thus enabling the detection and quantification of storage lipid levels in intact cells, tissue sections and even whole organisms^{12–15}. In particular, *C. elegans* are useful for lipid imaging studies owing to their transparency. Like mammals, *C. elegans* also store lipids in lipid droplets and the synthesis and degradation pathways of lipid molecules are highly conserved¹⁶. In this protocol, we will provide the working principle of SRS microscopy, its fundamental setup and describe the methods for its use in lipid imaging in *C. elegans*.

133 PROTOCOL:

135 1. Instrumental setup for Stimulated Raman Scattering Microscopy

137 NOTE: The SRS microscopy system is built upon a picosecond laser with pump integrated optical
138 parametric oscillator and a confocal laser scanning microscope. The oscillator provides two
139 picosecond pulse trains, including a Stokes beam at 1,064 nm and a pump beam tunable between
140 700–990 nm. Temporal and spatial overlapping of the two beams are achieved inside the laser.
141 A built-in electro-optic modulator (EOM) is designed specifically for SRS microscopy. This protocol
142 will focus on coupling the laser with the microscope, and the daily operation of this system
143 (**Figure 1**). The configuration of an SRS microscope system is evolving in the last decade. It should
144 be noted that the following description only represents one of the several configurations.

146 1.1. Feeding laser beams into the microscope

148 1.1.1. Set up the periscope to lift the beam from picosecond light source exit to IR laser input
149 port on the scanner of the microscope.

151 CAUTION: Read the laser system manual before operation and take all required precautions
152 because near infrared radiation generated by this Class IV laser system can cause severe harm to
153 the eyes and skin. Complete the trainings that are required by the institutional environmental
154 safety departments. Wear protective goggles and lab coat with cuffed sleeves.

156 1.1.2. First, set the pump beam at 750 nm and lower down the laser power to 50 mW, which can
157 be visually inspected, affiliating the alignment. Then, use a beam expander (**Figure 1**, L1 and L2)
158 to adjust the beam diameter to fit the back aperture of microscope objectives. Use a relay mirror
159 (**Figure 1**, M1) to guide the laser beam to the periscope (**Figure 1**, P1 and P2). The periscope will
160 lift the beam to the height of the scanner opening and serve as the steering mirror for alignment
161 as well.

163 1.1.3. Set the knobs on the periscope at the center of the tuning range and choose the initial
164 position and angle of each mirror such that the laser beam hits the center of the mirror,
165 approximately.

167 1.1.4. Perform the coarse alignment using an empty port on the objective turret and place the
168 power meter probe to measure the power of the transmitted light. Set the scanner of the
169 microscope at highest zoom (50x), and then measure the power of the transmitted light with the
170 focused laser beam spot. Optimize the knobs on each mirror of the periscope iteratively to
171 achieve the highest transmitted laser power.

173 1.1.5. Perform the fine alignment with the alignment tool. Put the fluorescence target alignment
174 cap on the empty objective seat and adjust the knobs of the bottom mirror of the periscope to
175 center the spot. Then, introduce the extension tube for the target and tune the knobs of the top
176 mirror of the periscope to center the spot.

177
178 1.1.6. Repeat steps 1.1.4 and 1.1.5 until the beam centers the target at both positions.
179

180 1.2. Connecting detection and electronics 181

182 1.2.1. Set up the SRS detection module. Place a flip mirror after the condenser to direct the
183 transmitted laser to the photodiode module. The module includes a telescope to relay and
184 change the beam size to fit the active area of the photodiode, as well as an optical filter to remove
185 the modulated Stokes beam and let the pump beam go through for demodulation.
186

187 1.2.2. Set up the electronics connection (**Figure 1**). The intensity of the Stokes beam is modulated
188 by a built-in EOM at 8 MHz inside the picosecond tunable laser. The modulation frequency output
189 from the laser is input into the lock-in amplifier as the reference frequency for demodulation.
190 Feed the output signal of the photodiode into the lock-in amplifier for demodulating the SRL.
191

192 NOTE: The one-box laser systems can be upgraded and thus their specifications can vary due to
193 different production date. The previous version of picosecond tunable laser system, used in this
194 protocol, has a Stokes beam of 1,064 nm, but the recent version has a Stokes beam of 1,032 nm.
195 The modulation frequency of EOM used in this protocol is customized to 8 MHz, but the default
196 modulation frequency for the recent system is 10 or 20 MHz.
197

198 1.2.3. Finally, feed the lock-in amplifier output into the analog box of the microscope to convert
199 electrical analog signal to digital signal. The system should be ready to process the SRS signal.
200

201 1.3. Optimize imaging conditions 202

203 1.3.1. Make a chemical sample for system alignment. For example, use dodecane to optimize the
204 microscope, because dodecane has a very strong SRS signal from the C-H bonds. Use a secure
205 seal to make a mini chamber; put 5 μ L of dodecane and cover it with a coverslip.
206

207 NOTE: Replace the dodecane sample once it looks unclear or presents debris, with a new sample
208 which could be used for alignment for 2–3 months.
209

210 1.3.2. Place the sample on the microscope stage. Properly focus on the liquid droplet. It can be
211 done faster by looking for the edge of the dodecane pad. Adjust the condenser for Kohler
212 illumination.
213

214 1.3.3. Set the imaging parameters. Check whether the delay stage is on the right numbers. Set
215 the pump beam wavelength to 816.7 nm. Using an IR sensor card and IR viewer, check whether
216 the pump beam path is still correct.
217

218 1.3.4. Set the power of the pump beam to 200 mW and the Stokes beam to 400 mW on the
219 picosecond system control panel.
220

NOTE: The laser throughputs from laser exit to post objective of the two beams are approximately 25% for pump and 14% for Stokes for this system setup. Therefore, a post objective power of pump beam is approximately 50 mW and for Stokes beam it is around 56 mW. The pulse width of the recent picosecond laser system has changed from 6 ps to 2 ps, therefore, the applied laser power should be even lower.

1.3.5. Set the lock-in amplifier gain to maximum. Open the shutter for both the beams, as well as the main shutter of the microscope.

1.3.6. Scan the sample and check the image on the computer screen. Change the display mode into **Hi-Lo** in the imaging software. Adjust the range to see an ~50% saturation. Check whether the saturation is centered in the image. If not, carefully adjust the steering mirrors of the Stokes beam in the picosecond tunable laser system to maximize the image intensity and to center the peak intensity.

1.3.7. Fine tune the delay stage and find the maximum SRS signal intensity from the dodecane sample. The system is then ready for use.

2. Preparation of *C. elegans* samples for SRS microscopy imaging

NOTE: As model organisms, *Caenorhabditis elegans* have been proven to be very useful for multiple imaging techniques. They are whole-body transparent, therefore various tissues can be imaged in the intact animal without the need for dissection. In *C. elegans*, neutral lipids are stored in lipid droplets located in the intestine, which consists of 20 epithelial cells located bisymmetrically around the intestinal lumen¹⁶. Hypodermal cells and oocytes also store lipids. The main form of storage lipids in *C. elegans* lipid droplets are the triacylglycerols (TAGs)¹⁷, which generate strong SRS signals due to the abundance of CH₂ bonds present in their fatty acid chains. This section will focus on how to prepare *C. elegans* samples for SRS microscopy imaging and quantification of their total intestinal lipid storage levels.

2.1 Preparation of the imaging slides

2.1.1. First, prepare 2% agarose solution in distilled H₂O, and make sure all the agarose melts before preparing the agarose pads.

2.1.2. Add one drop of warm 2% agarose onto a blank slide (approximately 100 µL) that is placed between support slides with two layers of laboratory tape and quickly place a second slide on top of the first slide. Gently press to create a thin, even agarose pad.

2.1.3. Set these slides aside in closed position and do not separate them until worms are ready to be mounted. Prepare fresh slides before each imaging session, as the pads will dry after 1 day.

NOTE: Use multi-well imaging chambers, if imaging several worms in high-throughput genetic screens¹⁸. Use live imaging setups to image spatio-temporal dynamics of lipid metabolism

through development and aging¹⁹.

2.2. Mounting the worms

2.2.1. Prepare the anaesthetizing agent by adding sodium azide or levamisole in M9 buffer to the final concentration of 100 mM or 1 mM, respectively.

NOTE: Use levamisole if the worms need to be recovered after imaging for genotyping after genetic screens.

CAUTION: Several anaesthetizing agents, including sodium azide and levamisole, are harmful if inhaled. Wear protective gloves and clothing, as well as use a chemical fume hood when preparing those working solutions.

2.2.2. Place a drop of anaesthetizing agent to a coverslip (approximately 4–5 μ L for 10–20 worms).

NOTE: Adjust the amount of the anaesthetizing agent according to the worm number to keep the worms as close to each other as possible. Using too much liquid for few worms may cause the worms to spread on the agarose pad.

2.2.3. Pick the worms to be imaged under the dissection microscopy and transfer them to the anaesthetizing agent droplet. After all the worms are added to the droplet, move them around using the worm picker and make sure they do not overlap with each other.

2.2.4 Separate the glass slides (from step 2.1) without perturbing the agarose pad.

2.2.5. Cover the worm droplet with the glass slide that has the agarose pad. Make sure the agarose pad is facing toward the worms. Alternatively, the anesthetizing aging droplet can also be added onto the agarose pad and worms can be covered with the coverslip.

2.2.6. Finally, mark the location of the worms using a permanent marker on the glass slide.

3. Image acquisition and analysis

3.1. Acquiring the images using the SRS microscope system (explained in section 1)

3.1.1. Before imaging any sample, determine the imaging parameters and verify SRS signals (as explained in section 1).

3.1.2. Mount the slide with the coverslip facing the objective lens. Turn on the bright field light source and direct it to the eyepiece to locate the worms.

NOTE: Use a 20x objective lens to image the lipid storage in the whole intestine. Consider using

a 60x objective lens to image hypodermal fat storage or to quantify lipid droplet size/number.

3.1.3. Bring the worms into focus and adjust the condenser position accordingly.

3.1.4. Turn off the bright field light source and switch to the laser scanning unit. Begin scanning the first worm at a fast scanning rate (e.g., 512 x 512 pixels), and adjust the fine focus to find the area of interest. For the first sample to be imaged, adjust the laser powers to the level where SRS signals are not saturated.

3.1.5. Switch to a slower scanning rate (e.g., 2 μ s/pixel) and higher resolution (e.g., 1024 x 1024 pixels) to obtain the SRS image.

NOTE: Keep the laser powers constant for every sample to be imaged during the imaging session.

3.1.6. Save the SRS image in the desired format that enables high resolution (such as a .tiff file). Depending on the imaging software and setup used, save the location of the first worm before moving to the next.

3.1.7. Complete imaging of all worms that were mounted on the slide. Turn off the shutter to block the laser beams. Do not turn off the laser source until all samples are imaged.

3.1.8. Remove the slide before placing the next sample slide. Repeat steps 3.1.3–3.1.7.

3.1.9. Once all the samples have been imaged, put the laser source on standby and turn off the associated equipment including the amplifier, the detector, the microscope and the computer.

3.2. Analysis of images using ImageJ

3.2.1. Open the image files (usually .tiff) to be quantified in ImageJ, by selecting the files and dragging and dropping them to the ImageJ window. Download the appropriate ImageJ plug-ins, if using specific Olympus microscope formats (such as .oib files).

3.2.2. Select the properties to be analyzed (**Analyze > Set Measurements**). For total lipid storage quantitation, select **Area**, **Min and Max Gray Value**, and **Mean Gray value**.

3.2.3. Use the polygon selection tool and outline the region of the worm intestine to be quantified. To measure the parameters selected in step 3.2.2, click on **Analyze > Measure**. A new window with the measurement results will pop-up. Repeat this step for all the open images.

3.2.4. Select the measurements for all the worms in a given genotype and copy/paste the results to a new spreadsheet. Repeat the steps 3.2.1–3.2.4 for all the genotypes in the same imaging session.

3.2.5. Select an area in the vicinity of the worm that does not have any SRS signal, to quantify as

background. Make sure the area selected for background measurement does not contain any bacteria or debris that could also give an SRS signal.

3.2.6. Subtract the background mean gray value from the measurements of each individual worm. Calculate the average and standard deviation of the background subtracted mean gray values from all of the worms in a given genotype/test group. Normalize this value to the average of the control group.

3.2.7. Finally, perform the appropriate statistical analysis using mean the gray value as a measure for lipid levels in each worm. Typically, use Student's *t*-test for two groups and use one-way ANOVA with an appropriate multiple comparison test for more than two groups.

NOTE: When selecting images for figure presentation, ensure that those selected images have the same pixel intensity distribution. Set the brightness and contrast to the same values for all the images in the same figure (**Image > Adjust > Brightness and Contrast**).

REPRESENTATIVE RESULTS:

Insulin signaling is an important endocrine pathway that impacts development, reproduction, lifespan, and metabolism. In worms, insulin signaling consists of about 40 insulin-like peptide ligands, insulin-like growth factor receptor ortholog DAF-2, downstream PI3K/AKT kinase cascade, and the FoxO transcription factor ortholog, DAF-16²⁰. *daf-2* mutants, that lack the insulin receptor, have more lipid droplets in their intestine, than the worm lipid storage tissue^{21,22}. Using SRS microscopy, we quantified the intestinal lipid levels in age-synchronized wild-type worms and *daf-2* mutants during adulthood. Consistent with the previous results, we found that *daf-2* mutants have higher lipid levels^{23,24} (**Figure 2A**). We observed a decline in lipid levels in wild-type worms starting day 1 until day 9 of adulthood (**Figure 2A**). However, the lipid levels in *daf-2* mutants increased until they were 5-days-old and then they were constant around the level of 2.5–3.0 fold higher than the wild-type (**Figure 2A**).

DAF-16, the *C. elegans* FoxO transcription factor ortholog, orchestrates most of the downstream functions of the insulin signaling pathway. *daf-16* null mutations suppress several phenotypes observed in *daf-2* mutants, including fat accumulation. *daf-16* genomic locus encodes for 8 different transcripts (*daf-16a-h*), that are generated by either alternative transcription start site, or alternative splicing²⁰. Among those, *daf-16a*, *daf-16b* and *daf-16d/f/h* are the most abundant transcripts and they encode for proteins with different N termini. Studies with isoform-specific mutations and transgenes suggested that DAF-16A and DAF-16D/F/H isoforms regulate dauer development and lifespan^{24,25}, whereas DAF-16B is important for pharyngeal remodeling during dauer formation²⁶. In this study, using SRS microscopy, we investigated the isoform specificity of DAF-16 in regulating intestinal lipid storage. We found that *daf-16* inactivation suppresses the increase in lipid levels of *daf-2* mutants (**Figure 2B,C**). Expressing *daf-16a* or *daf-16b* isoform in the *daf-2; daf-16* double mutants does not restore the lipid levels. However, the expression of *daf-16d/f/h* isoform is sufficient to restore the lipid levels in *daf-2; daf-16* to the levels observed in *daf-2* single mutants (**Figure 2B,C**). These results revealed that specifically DAF-16D/F/H isoforms modulates lipid metabolism in *daf-2* mutant worms. This is also consistent with the

results from a recent study that showed olfactory regulation of lipid storage is also mediated through the transcriptional activities of DAF-16D/F/H²⁷.

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of the SRS microscope system. The all-in-one laser provides temporally and spatially combined pump and Stokes beams, built-in electro-optic modulator (EOM) and reference frequency for lock-in amplifier. The combined beam is expanded by the telescope (L1 and L2), relayed by the relay mirror (M1) and lifted by the periscope (P1 and P2), and then sent into the scanner of the microscope. The combined beam is scanned by the scanning unit (SU) in the microscope and focused on the sample. Transmitted light is collected by the condenser. A filter will remove the Stokes beam and leave the pump beam to be detected by the photodiode (PD). The gray dashed arrows show the connection between the electronics.

Figure 2: Reduced insulin signaling in *C. elegans* leads to increased lipid storage. (A) Using SRS microscopy, intestinal lipid levels of wild-type and *daf-2* mutants worms are quantified in day 1, 3, 5, 7 and 9 of adulthood. In wild-type worms, the lipid levels decline over time, whereas *daf-2* mutants accumulate lipids until day 5 and they have about 2.7 fold more lipids compared to wild-type worms. (B) *daf-16* deletion suppresses the increased lipid levels in *daf-2* mutants. Restoring expression of the *daf-16d/f/h* isoform in *daf-2; daf-16* double mutants is sufficient to increase lipid levels to *daf-2* mutants' levels, whereas restoring expression of *daf-16a* or *daf-16b* isoforms is not. *** $p < 0.001$, n.s. not significant by one-way ANOVA with Bonferroni's multiple comparisons test. (C) Representative SRS images. Yellow pixels indicate higher lipid levels. Quantified intestinal area is shown in dashed lines. Scale bar is 40 μm .

DISCUSSION:

In defense against obesity and its associated metabolic disorders, important research efforts have been implemented to better understand the regulatory mechanisms of lipid homeostasis. For quantitative detection of lipid molecules in biological samples, label-free imaging by SRS microscopy has been proven to be a reliable alternative to biochemical assays and other staining methods. Our group and others have revealed novel biological mechanisms in lipid metabolic regulation by combining the use of *C. elegans* and SRS microscopy^{12,18,28-31}. In this protocol, using SRS microscopy we demonstrated that downregulating insulin signaling in worms leads to an increase in total intestinal lipid levels. We observed that starting day 1 of adulthood, lipids accumulate in the intestine of the *daf-2* mutants, which have loss-of-function mutation in the insulin receptor gene, whereas in wild-type worms intestinal lipid levels decline. Additionally, consistent with previous studies^{23,24}, we found that *daf-16* inactivation can fully suppress the lipid storage increase in *daf-2* mutants and among the different *daf-16* isoforms, *daf-16d/f/h* isoform specifically regulates lipid homeostasis. As previous studies suggested, the difference in total intestinal lipid storage levels in *daf-2* mutants is likely due to a combination of factors: increased *de novo* lipid synthesis³², decreased lipid utilization³³, as well as reduced reproduction, and yolk accumulation³⁴. Recent advances in coherent Raman spectroscopy techniques (SRS and CARS) would help further elucidate the mechanisms of how insulin signaling contributes to the regulation of different lipid storage types in distinct tissues, including the intestine, hypodermis

and oocytes.

SRS microscopy has been utilized to image not only lipids, but also other biomolecules, including DNA³⁵, RNA¹⁴, proteins¹³, and glucose³⁶. It has been implemented to provide important insights into many aspects of biology such as cell biology, microbiology, cancer biology, and developmental biology¹¹. More recently, the use of bioorthogonal tags (e.g., alkyne and deuterium tags), to minimally label the molecules of interest, further enhanced the detection sensitivity and specificity. These minimal vibrational tags do not disrupt the chemical properties of the molecule, but provide specific chemical contrast, as their Raman signal is within the “silent window” of the Raman spectrum^{14,37}. In addition to improving sensitivity and specificity, achieving super-resolution SRS imaging is also an exciting area of research. Several research groups also have efforts in minimizing the equipment size by creating hand-held SRS microscope³⁸. Thus, application of SRS imaging to biomedical research has opened up new venues for technological and chemical improvements to advance lipid imaging.

Compared to traditional methods of lipid quantitation, SRS microscopy has several advantages. First of all, it is performed in a label-free manner and even if a label has to be used, the label can be as small as two atoms. Therefore, it also does not suffer from the photobleaching problem as other fluorescent tagging and staining methods. Secondly, the capability of live imaging using whole organisms enables tracking the dynamics of specific metabolites^{29,39,40}. Finally, SRS microscopy permits higher degree of multiplexing and therefore can be implemented to image multiple biomolecules in distinct cellular compartments simultaneously. Hyperspectral SRS/CARS microscopy holds great future in exploring the metabolic dynamics of several distinct biomolecules, as well as different types of lipid storage compartments such as yolk particles in *C. elegans*⁴¹ or cholesteryl ester and TAG rich lipid droplets in different mammalian cells and tissues³¹. On the other hand, SRS microscopy is facing limitations such as high cost in hardware and lack of full commercial availability. Additionally, it requires expertise to maintain and run the system, which can complicate its adoption. The developments in new light source and system integration are improving the situation. Fiber-based low cost laser source and fully integrated systems offer new opportunities in commercialization and will help reduce the cost in the future^{11,42,43}. In addition, despite the new technological approaches to improve the imaging speed and spatial resolution of SRS imaging, the limited detection sensitivity may continue to hinder the capability to probe metabolites of low abundance. Overall, the use of SRS microscopy in biomedical research has grown tremendously in the last decade. SRS imaging will further benefit from the technological improvements in detection sensitivity, spatial resolution and multiplexing capability, and ultimately expand its use in quantitative monitoring of biomolecules, especially lipids.

ACKNOWLEDGMENTS:

This work was supported by NIH grants R01AG045183 (M.C.W.), R01AT009050 (M.C.W.), R01AG062257 (M.C.W.), DP1DK113644 (M.C.W.), March of Dimes Foundation (M.C.W.), Welch Foundation (M.C.W.), and by HHMI investigator (M.C.W.). We thank the Caenorhabditis Genetics Center (CGC) for *C. elegans* strains.

DISCLOSURES:

The authors have nothing to disclose.

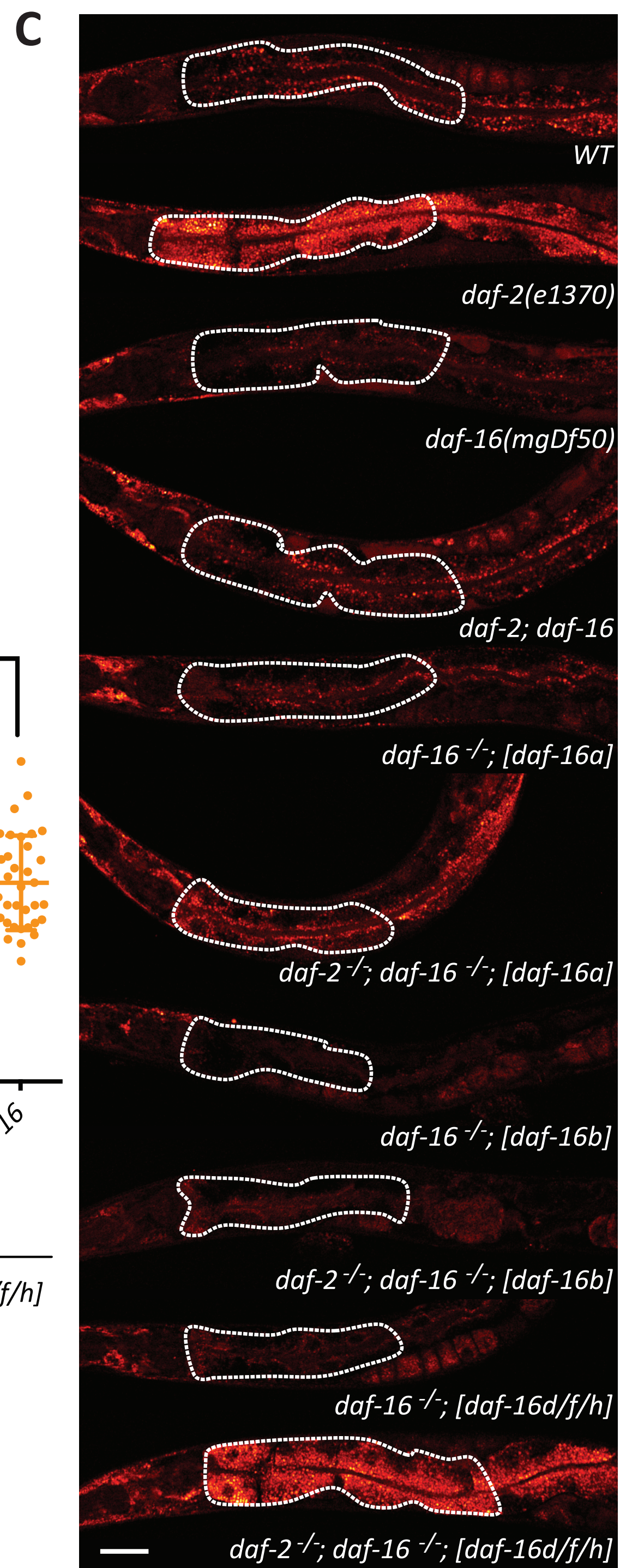
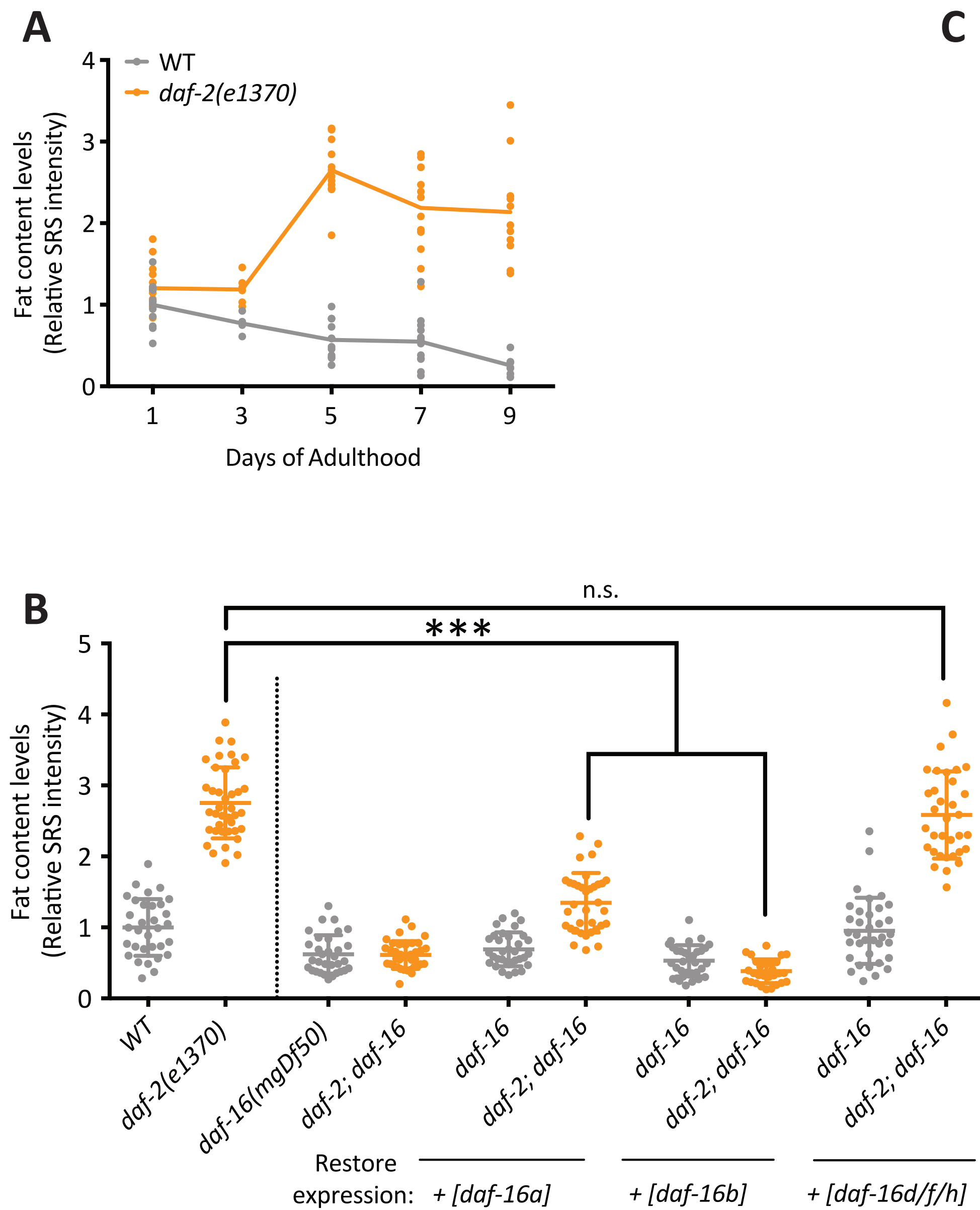
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
A/D converter	Olympus	Analog Unit	
Agarose	GeneMate	3119	For making agarose pads
Alignment tool - adapter	Thorlabs	SM1A4	For mounting the tool on scope
Alignment tool - target	Thorlabs	VRC2SM1	For viewing IR laser
Alignment tool - tube	Thorlabs	SM1L40	Length can vary
Autocorrelator (Optional)	APE	pulseCheck	
Bandpass filter	minicircuits	BBP-21.4+	For signal with modulation frequency filtering
BNC cables			
Dissection microscope	Nikon	SMZ800	For handling and picking worms for imaging
Dodecane	Sigma-Aldrich	44010	Used for calibration of the SRS signal
	Chroma		
Filter	Technology	890/220 CARS	For removing Stokes beam
General purpose laboratory labeling tape	VWR	89097	For making agarose pads
Glass coverslips	VWR	48393-106	For covering worms for imaging
Glass microscope slide	VWR	16004-422	For making agarose pads
Laser scanning microscope	Olympus	FV3000	
		L1: AC254-050-B	
Lens	Thorlabs	L2: AC254-075-B	For beam expander
Lock-in amplifier	Zurich	HF2LI	
Lowpass filter	minicircuits	BLP-1.9+	For power supply noise suppression
Mirrors	Thorlabs	BB1-E03	For relay and periscope
		UPlanSAPO 20x	
		0.75, UPlanSAPO	
Objective	Olympus	60XW 1.20	
Photodiode	Thorlabs	FDS1010	
Picosecond laser source	APE	picoEmerald	
Power supply	TEKPOWER	TP1342U	For photodiode, reversed 50V voltage
Sodium azide	Sigma	S2002	For anaesthetizing the worms
Worm picker	WormStuff	59-AWP	



Baylor
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Medicine

09/08/2020

Dear Dr. Nguyen,

Thank you very much for sending us the editorial and reviewers' comments. We are pleased that the reviewers raised mostly minor concerns. Based on their suggestions, we have, 1) updated the technical details about the SRS system setup and laser safety, 2) mentioned this protocol describes only one of several SRS system configurations, 3) elaborated more on the different lipid storage types in *C. elegans* and on the recent advances with capabilities to detect these. We have addressed all the comments in our point-by-point response attached below. Please let us know if you would have any questions and/or would need any further information.

With best wishes,



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POINT-BY-POINT RESPONSE

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript for spelling and grammar errors.

2. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We changed the protocol steps and notes to ensure they are only action items.

3. Please note that anesthetization cannot be filmed in the protocol.

We will only show the step where the worms are picked into the anaesthetizing agent droplet on the coverslip using a dissecting microscope. The worms will not be seen in the video. Please let us know if we still need to remove this step from the highlights for the video.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

SRS microscopy has become a very useful tool for biomedical research. In this manuscript, the authors present the working principles and the detailed setup of the SRS microscope, and also provide methods for its use in quantifying lipid storage at distinct developmental timepoints of wild-type and insulin signaling deficient mutant *C. elegans*. Generally, it is a very good protocol for those who are interested in building their own SRS microscope or using SRS microscope to study lipid metabolism in *C. elegans*.

We appreciate the reviewer finding our protocol a good resource for those who are interested in utilizing SRS microscope in their research.

Major Concerns:

None.

Minor Concerns:

1. It should be noted somewhere in the manuscript that the protocol presented here is just one of the protocols that can be followed to build a SRS microscope.

We thank the reviewer for this suggestion to mention that this protocol should be used with an SRS microscope system that utilizes a picosecond laser with pump integrated optical parametric oscillator.

We have now added additional information in Protocol 1 that there could be technical differences with each SRS system setup (lines 151-153 and lines 201-205).

2. As described in the APE website, the wavelength of picoEmerald should be around 1030nm instead of 1064nm, and the modulation frequency of EOM should be 10MHz or 20MHz instead of 8MHz. Please double check these parameters.

We thank the reviewer for bringing this important point to our attention. The APE company has upgraded the picoEmerald one-box laser system. Therefore, specifications can vary due to different production date. The older version of picoEmerald, used in this protocol, has a Stokes beam of 1064 nm, but the new version has a Stokes beam of 1032 nm. Moreover, the modulation frequency of EOM used in this protocol is customized to 8 MHz, but the default modulation frequency for the new version picoEmerald is 10 or 20 MHz. We have added this information as a note after protocol step 1.2.2 (lines 201-205).

3. I'm not sure why there are many paragraphs highlighted in yellow color in the manuscript.

As per the author guidelines of JoVE, we used the yellow highlighting to indicate the essential parts of the protocol to be filmed.

Reviewer #2:

Manuscript Summary:

The authors describe the use of stimulated Raman scattering to assess lipid storage in *C. elegans*.

Major Concerns:

The way the authors describe *C. elegans*' fat in this manuscript is a little bit misleading. Not all the *C. elegans*' fat is the same. For example, the yolky fat, analogous to human low-density lipoprotein fat, is destined to the progeny but not utilized by the parent worms. The production of yolk (vitellogenesis) is also a critical modulator that regulates the reproduction and aging in *C. elegans*, but the regulation pathways could be different from obesity-related metabolic disorders [Current Biology 28, 2544-2556 (2018); Front. Physiol. 10:1067.doi: 10.3389/fphys.2019.01067 (2019)]. However, both histochemical lipid staining and single-frequency SRS/CARS cannot tell the difference between the fats for progeny (yolk) and for somatic maintenance [Nat. Chem. Biol. doi.org/10.1038/s41589-020-0565-2 (2020)]. The authors should mention this limitation. The authors should also point out the complexity of these fats but not simply try to draw parallels between all the *C. elegans*' fat content (particularly the intestinal fat) and adiposity in mammals.

We thank the reviewer for bringing these publications to our attention. We are sorry that the reviewer found our description about *C. elegans* fat storage misleading. Studying the difference between lipid storage types, including the intestine and the hypodermis, is quite exciting, however it is out of the scope of this protocol. In this protocol, our goal is to explain how *C. elegans* samples are used for quantifying total intestinal lipid storage by SRS imaging. To prevent any further misunderstanding, we now state this goal specifically in Protocol 2 (line 257-258). In the Discussion section, we also included an explanation about the changes in *daf-2* mutants' intestinal lipid storage levels that are likely due to a combination factors, including the overall lipid synthesis and utilization, as well as the changes in yolk

production and accumulation (lines 450-453). Additionally, we mentioned that advances in hyperspectral SRS/CARS will enable us to investigate the distinct types of lipid storage particles in different tissues (lines 477-481).

Minor Concerns:

The laser power, pump = 200mW, Stokes=400mW, seems too high. What is the exactly power of both beam on the focus plane? Typically, <20mW on the sample works well enough to obtain sufficient coherent Raman signal. The power should be even less because the author's laser system has high peak power due to the low repetition rate.

We thank the reviewer for this comment. We have measured the beam power on the focus plane and it is ~50 mW for the pump beam and ~56 mW for the Stokes beam. We have added these measurements as a note in Protocol 1, after step 1.3.4 (lines 231-235).

Reviewer #3:

Manuscript Summary:

The reviewed article presents Stimulated Raman Scattering Microscopy as a method to quantify lipids in a label-free manner and in vivo in *Caenorhabditis elegans*. Results of the experiment were previously published. The designed experiment is well-selected to demonstrate applicability of SRS microscopy for fast and reliable quantification of lipids in vivo in a model organism (particularly attractive for this kind of analysis due to its transparency and ease of genotyping). I find the topic clearly interesting and well-chosen to popularize SRS that is yet a niche method despite its great potential in biological sciences. The article is well written, therefore I have only minor remarks (below).

We appreciate the reviewer finding our protocol “interesting and well-chosen”.

Minor Concerns:

Introduction:

The introduction contains all necessary information that a reader needs to understand the purpose of the experiment, limitations of other techniques for reliable lipid quantification and imaging, and basics of both Raman and SRS microscopy.

"The molecular vibration will experience an enhanced transition (...)" I would rather say: "The molecule will experience an enhanced transition (...)".

We thank the reviewer for this comment and have made the suggested change (line 116).

Protocol:

The protocol is clear and detailed.

1.1.1 Could the authors explain the term "scanner import"?

We thank the reviewer for this feedback. We now changed the term to “IR laser input port” (line 157).

1.1.2. "Firstly, set the pump beam at 800 nm, which can be visually inspected" Is the beam visible at 800

nm?

No information about necessary precautions due to use of laser beams are included (protective eyewear?).

We thank the reviewer for these suggestions. It is correct that the picosecond 800 nm pump beam is invisible for alignment. For alignment purposes, we need to tune the laser wavelength down to 750 nm and lower down the laser power to 50 mW for safety and visual inspection of the optical path. We now changed this in the main text (line 165). Once the wavelength is switched to 816.7nm, the pump beam path can be checked using an IR sensor card or IR viewer (line 225-226). Moreover, we have added more details on laser safety in Protocol 1, after step 1.1.1 (lines 160-163).

3.2.2. Are the differences in the measured area taken into account?

When quantifying the *C. elegans* intestinal lipid levels in SRS microscopy images, we select the first 3-4 pairs of anterior intestinal cells in each worm and measure the average pixel intensity. Although the intestinal area selected in each worm can be different in size, total pixel intensity will be averaged to the area. Since the lipid droplets are homogenously distributed in *C. elegans* intestinal cells, the average SRS signal intensity is a reliable measure for lipid levels. We thank the reviewer for this comment and we have added this explanation to image analysis section, step 3.2.6 (lines 367-370).

Representative results:

I suggest to develop/simplify this part to make it more understandable for readers without the biological background.

We thank the reviewer for this feedback and we have simplified the results section.

Discussion:

Discussion summarizes well advantages of SRS and its perspectives in biosciences.

We thank the reviewer for the feedbacks that improved our manuscript.