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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **38**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Tao Chen**: Stimulated Raman scattering microscopy is a chemical imaging technology that enables the rapid and quantitative detection of lipids and allows the tracking of lipids in live animals in a label-free manner **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Dinghuan Deng**: The most important advantage of SRS microscopy over traditional lipid detection techniques is that it is label-free and therefore is not as susceptible to photobleaching as other fluorescence and staining methods **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Sena Mutlu**: The use of SRS microscopy in conjunction with the genetic and biochemical tools available for model organisms, particularly *Caenorhabditis elegans*, provides a powerful framework for studying novel regulators of lipid biology and metabolism **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Stimulated Raman Scattering (SRS) Microscopy Instrument Setup

2.1. To feed laser beams into the SRS (S-R-S) microscope, first set the periscope to lift the beam from a picosecond light source exit to the infrared laser input port on the scanner of the microscope **[1]** and open the laser controller software to start the lasers **[2].**

2.1.1. WIDE: Talent setting periscope
2.1.2. SCREEN: screenshot_1: 00:03-00:08

2.2. Lower the pump laser power to 50 milliwatts and set the pump signal to 750 nanometers **[1]**. Use a beam expander to adjust the beam diameter to fit the back aperture of the microscope objectives **[2]**.

2.2.1. SCREEN: screenshot_3: 00:06-32 *Video Editor: please speed up*
2.2.2. Talent testing expander attachment on the to aperture

2.3. Use two relay mirrors, M1 and M2, to guide the laser beam to the periscope **[1]** and set the knobs of the periscope at the center of the tuning range **[2]**.

2.3.1. Talent guiding laser beam to periscope
2.3.2. Talent testing the knobs are in the center of range

2.4. Select the initial position and angle of each mirror such that the laser beam approximately hits the center of the mirror to launch the beam into the microscope **[1]** and use an empty port on the objective turret of the microscope to perform the coarse alignment **[2]**.

2.4.1. Talent setting angle(s) of M1, M2, P1 and P2
2.4.2. Talent using input port and objective port for coarse alignment

2.5. Place the power meter probe at the objective port to measure the power of the transmitted light **[1]** and set the scanner of the microscope at the highest zoom, 50X **[2]**.

2.5.1. Talent placing probe to measure the power of the transmitted light NOTE: 2.5.1a see reading
2.5.2. Talent setting scanner at highest zoom 50X to focus the laser beam spot on the probe

2.6. Measure the power of the transmitted light with the focused laser beam spot **[1]** and optimize the knobs of mirrors, M1 and M2, iteratively to achieve the highest transmitted laser power **[2]**.

2.6.1. Talent measuring the power of transmitted light NOTE: This and next shot together
2.6.2. Talent optimizing knob(s) of M1 and M2

2.7. Use the alignment tool to perform the fine alignment of the transmitted light to make sure the light is passing through the center of the objective **[1]** and place the fluorescence target alignment cap on the empty objective seat **[2]**.

2.7.1. Talent performing alignment
2.7.2. Talent placing cap onto the objective seat

2.8. Adjust the knobs of the first steering mirror, M1, to center the laser beam spot **[1]** and introduce the extension tube with the alignment cap on the other end, to an empty objective seat for monitoring the laser beam spot **[2]**.

2.8.1. Talent adjusting knob(s) of M1 NOTE: 2.8.1a target moving
2.8.2. Talent introducing the extension tube of the alignment tool NOTE: 2.8.2a show beam off target

2.9. Then tune the knobs of the second steering mirror, M2, to center the laser beam spot again on the other end of the extension tube **[1-TXT]**.

2.9.1. Talent tuning knob(s) of M2 *Videographer: Important/difficult step* **TEXT: Repeat coarse and fine alignments until the laser beam spot centers target at both positions**

2.10. To set up the SRS detection module, place a beam splitter cube after the condenser to direct the transmitted laser to the photodiode module **[1]**.

2.10.1. Talent placing beam splitter cube after condenser

2.11. To set up the electronics connection, use a built-in electro-optic modulator at 20 megahertz inside the picosecond tunable laser to modulate the intensity of the Stokes beam **[1]** and feed the output signal of the photodiode into the lock-in amplifier for demodulation of the stimulated Raman loss **[2]**.

2.11.1. SCREEN: screenshot_4: 00:04-00:15
2.11.2. SCREEN: screenshot_5: 00:20-01:25 *Video Editor: please speed up*

2.12. Then feed the lock-in amplifier output into the analog box of the microscope to convert the electrical analog signal to a digital signal **[1]**.

2.12.1. Talent feeding output to analog box NOTE: 2.12.1a show input port

3. Imaging Condition Optimization

3.1. To optimize the imaging conditions, add 5 microliters of oleic acid to a mini-microscope slide chamber **[1]** and cover the sample with a coverslip **[2]**.

3.1.1. WIDE: Talent adding sample to slide
3.1.2. Talent placing coverslip onto slide

3.2. Place the sample onto the microscope stage **[1]** and use the edge of the pad to locate and focus on the droplet **[2]**.

3.2.1. Talent placing sample onto stage
3.2.2. SCREEN: screenshot_6: 00:05-00:27 *Video Editor: please speed up*

3.3. Adjust the condenser for Kohler illumination **[1]** and use an infrared sensor card and infrared viewer to check whether the pump beam path is still correct **[2]**.

3.3.1. Talent adjusting the condenser
3.3.2. Talent checking pump beam path with infrared sensor card

3.4. Confirm that the delay stage is set to 0 femtosecond and set the pump beam wavelength to 795.8 nanometers **[1]**. Set the power of the pump beam to 50 milliwatts if necessary and set the Stokes beam to 100 milliwatts. Open the shutter for both the beams as well as the main shutter of the microscope **[1]**.

3.4.1. SCREEN: screenshot_7: 00:04-00:20 *Video Editor: please speed up*
3.4.2. SCREEN: screenshot_7: 01:56-02:20 *Video Editor: please speed up*

3.5. Scan the sample and check the image on the computer screen **[1]**.

3.5.1. SCREEN: screenshot_6: 00:28-00:44 *Video Editor: please speed up*

3.6. Change the display mode to **Hi-Lo** and adjust the range to see an approximately 50% saturation. Check whether the saturation is centered in the image **[1]**, carefully adjusting the steering mirrors inside the picosecond tunable laser system for optimized overlap of the pump and the Stokes to maximize the image intensity and to center the peak intensity as necessary **[2]**.

3.6.1. SCREEN: screeshot_6: 00:45-01:15 *Video Editor: please speed up*
3.6.2. Talent adjusting mirrors *Videographer: Important step*

4. *C. elegans* Sample Preparation

4.1. Before each imaging session, add 100 microliters of warm 2% agarose to a clean glass slide placed between support slides with two layers of laboratory tape **[1]** and quickly place a second slide on top of the first slide **[2]**.

4.1.1. WIDE: Talent adding agarose to slide
4.1.2. Talent placing slide onto slide NOTE: 4.1.2 – 4.2.2. shot together

4.2. Gently press to create a thin, even agarose pad **[1]** and allow the agarose to cool **[2]**.

4.2.1. Slides being pressed
4.2.2. Talent placing slides aside

4.3. To mount the worms for imaging, place a 4-5 microliter drop of anesthetic agent per 10-20 worms onto a coverslip **[1-TXT]** and use a dissection microscope to pick the worms to be imaged **[2]**, placing the worms onto the droplet of anesthesia as they are collected **[3]**.

4.3.1. Talent adding drop to coverslip **TEXT: See text for anesthetic agent suggestion details**
4.3.2. Talent at microscope, picking worms
4.3.3. Worm(s) being placed into droplet

4.4. When all of the worms have been collected, cover the worms with the glass slide with the agarose pad **[1-TXT]**.

4.4.1. Pad being placed onto worms **TEXT: Indicate worm location with permanent marker**

5. Image Acquisition and Analysis

5.1. For imaging, mount the worm sample with the coverslip facing the objective lens **[1]** and direct the bright field light source to the eyepiece to locate the worms **[2]**.

5.1.1. WIDE: Talent placing sample onto stage
5.1.2. Talent directing light source to eyepiece

5.2. Bring the worms into focus **[1]** and adjust the condenser position accordingly **[2]**.

5.2.1. Talent bringing worms into focus
5.2.2. Talent adjusting condenser

5.3. Adjust the laser powers by setting the pump laser power to 200 milliwatts and the IR power to 400 milliwatts if necessary **[1]**. Set the lock-in amplifier for demodulation of the worm SRS signal **[2]**.

5.3.1. SCREEN: screenshot_8: 02:13-02
5.3.2. SCREEN: screenshot_9: 00:04-00:41 *Video Editor: please speed up*

5.4. Begin scanning the first worm at fast scanning rate, adjusting the fine focus to find the area of interest **[1]**.

5.4.1. SCREEN: screenshot_10: 00:08-00:23 *Video Editor: please speed up*

5.5. When the signal has been optimized, switch to a slower scanning rate and a higher pixel resolution to obtain the SRS image **[1]** and save the image in a format that enables high resolution **[2-TXT]**.

5.5.1. SCREEN: screenshot_10: 00:34-00:58 *Video Editor: please speed up*
5.5.2. SCREEN: screenshot_10: 01:00-01:17 *Video Editor: please speed up* **TEXT: Repeat for each worm**

5.6. When all of the samples have been imaged, place the laser source on standby **[1]** and turn off all of the associated equipment **[2]**.

5.6.1. Talent placing laser on standby
5.6.2. Talent turning off equipment

6. Images Analysis

6.1. To analyze the images, open the files in ImageJ **[1]** and select **Analyze** and **Set Measurements** to select the properties to be analyzed **[2]**.

6.1.1. WIDE: Talent opening file, with monitor visible in frame
6.1.2. SCREEN: screenshot_11: 00:36-00:52 *Video Editor: please speed up*

6.2. Use the polygon selection tool to outline the region of the worm intestine to be quantified **[1]** and click **Analyze** and **Measure** again. The measurements will appear in the Results window **[2]**.

6.2.1. SCREEN: screenshot_11: 00:58-01:16 *Video Editor: please speed up*

6.2.2. SCREEN: screenshot_1:1: 01:18-01:20

6.3. When all of the worms have been outlined, copy and paste the measurements for each of the worms in a given genotype into a spreadsheet **[1]**.

6.3.1. SCREEN: screenshot_11: 02:17-02:29 **TEXT: Repeat for all groups**

6.4. For the background measurement value, aelect an area in the vicinity of the worm that does not have any SRS signal **[1]** and subtract the background mean gray value from the measurement for each individual worm to calculate the average and standard deviation of the background subtracted mean gray values from all of the worms in a given genotype or test group **[2]**.

6.4.1. SCREEN: screenshot_11: 05:35-05:40 **TEXT: Repeat for at least 5 images**
6.4.2. SCREEN: screenshot_11: 06:07-06:27 *Video Editor: please speed up*

6.5. These values can then be normalized to the average of the control group **[1]**.

6.5.1. SCREEN: screenshot_11: 06:28-06:55 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.9., 3.6.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.9. Proper beam alignment is essential for SRS imaging. Therefore, mirrors have to be adjusted several times to ensure proper alignment and thus maximum SRS signal. Re-alignment could be necessary everytime SRS microscope is used.

Results

7. Results: Representative Effects of Insulin Signaling on Lipid Storage in *C. elegans*

7.1. In this representative analysis **[1]**, the intestinal lipid levels in age-synchronized wild-type worms **[2]** and *daf-2* (daff-two) mutants lacking the insulin receptor were quantified during adulthood **[3]**.

7.1.1. LAB MEDIA: Figure 2A
7.1.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize grey data line*
7.1.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize orange data line*

7.2. As expected, a decline in lipid levels is observed in wild-type worms starting at day 1 and continuing until day 9 of adulthood **[1]**. The lipid levels in *daf-2* mutants, however, increase until they are 5 days old **[2]**, at which point they remain constant at about 2.5-3-fold higher than the wild-type **[3]**.

7.2.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize decline in grey data line*
7.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize orange data line from d1-d5*
7.2.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize orange data line from d5-d9*

7.3. *Daf-16* inactivation suppresses the increase in lipid levels of *daf-2* mutants **[1]**.

7.3.1. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize first dat-2; daf-16 orange data cluster and top daf2; daf-16 outline*

7.4. Expression of the *daf-16a* or *daf-16b* isoform in the *daf-2 daf-16* double mutants does not restore the lipid levels **[1]**.

7.4.1. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize second and third daf-2; daf-16 orange clusters and daf 2-/-; daf 16-/- [daf-16a] and [daf-16b] outlines*

7.5. The expression of the *daf-16d-f-h* isoform, however, is sufficient to restore the lipid levels in *daf-2 daf-16* double mutants **[1]** to the levels observed in *daf-2* single mutants **[2]**.

7.5.1. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize last orange cluster and bottom outline*

7.5.2. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize first orange cluster and second outline*

7.6. These results indicate that the DAF-16D-F-H isoform specifically modulates lipid metabolism in *daf-2* mutant worms **[1]**.

7.6.1. LAB MEDIA: Figures 2B and 2C

Conclusion

8. Conclusion Interview Statements

8.1. **Dinghuan Deng**: It is important to use the same laser power for pump and Stokes beams throughout the imaging session and to include a control group in every session for consistent quantification **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.1.)

8.2. **Sena Mutlu**: In addition to quantifying total lipid storage, SRS microscopy can be coupled with bioorthogonal tags such as deuterium and implemented to visualize the dynamics of lipid incorporation, synthesis, and degradation **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

8.3. **Tao Chen**: SRS microscopy permits multiplexing and can be implemented to image multiple biomolecules in different subcellular compartments. So-called hyperspectral SRS microscopy has a great future in exploring metabolic dynamics **[1]**.

8.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera