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Title: Simultaneous Label-Free Autofluorescence Multi-Harmonic 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 something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Lengths
Number of Steps: 22
Number of Shots: 54

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Kevin K.D. Tan:** Simultaneous label-free autofluorescence multiharmonic microscopy captures unique information in biological samples by measuring nonlinear interactions with light. In our lab, we use this technique to study life and disease at the microscopic scale.

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

What are the most recent developments in your field of research?

1.2. **Stephen A. Boppart:** We observe a shift toward label-free multimodal imaging, offering richer insights into cellular and tissue structures and dynamics while avoiding toxicity and enabling complementary contrasts and correlations across modalities.

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

What are the current experimental challenges?

1.3. **Alejandro De la Cadena:** State-of-the-art label-free nonlinear microscopes acquire one signal at a time, risking misalignment, photodamage, and loss of concurrent processes. SLAM overcomes these issues by enabling strictly coregistered multimodal contrasts, ensuring reliable spatial and temporal data.

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

What advantage does your protocol offer compared to other techniques?

1.4. **Alexander Ho:** This technique enables simultaneous excitation and detection of four channels in a multiphoton microscope, using a photonic crystal fiber to broaden pulses for SHG, THG, and two- and three-photon fluorescence.

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

How will your findings advance research in your field?

1.5. **Edita Aksamitiene:** SLAM captures multiplexed metabolic, structural, and dynamic information without alignment artifacts or labels, enabling standardized quantitative tissue analysis. Its applications span fundamental biology, translational research, and biomarker discovery in oncology, neurodegeneration, and tissue regeneration.

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

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois

2. Pulse Characterization and Autocorrelation Setup for SLAM Microscopy

Demonstrators: Kevin K.D. Tan, Alexander Ho

2.1. To begin, use a spectrometer or optical spectrum analyzer to measure the laser after the parabolic mirror and verify the supercontinuum generation of the photonic crystal fiber **[1]**. Rotate the half-wave plate after the polarizing beam splitter to optimize the width of the supercontinuum **[2]**.

2.1.1. WIDE: Talent holding the optical spectrum analyzer probe at the laser path following the parabolic mirror.

2.1.2. Talent rotating the half wave plate (HWP2) mounted after the beam splitter.

2.2. Turn on the microscopy autocorrelator **[1]**. In the software, set the detector to the internal detector and adjust the scan range to 5 picoseconds to capture the full width of the pulse autocorrelation **[2]**.

2.2.1. Talent pressing the power switch on the autocorrelator controller.

2.2.2. SCREEN: 68637_screenshot_1.mp4 00:00-00:13

2.3. Unblock the laser and align the autocorrelator to the beam exiting the pulse shaper until a signal appears **[1]**.

2.3.1. Talent removing a beam block and adjusting mirrors to guide the laser beam into the autocorrelator.

2.4. Ensure that shutter 2 is closed **[1]**. Then apply the appropriate immersion media, such as water or oil, to the objective lens **[2]**. Place the autocorrelator external detector on the microscope stage and position it directly above the objective **[3]**.

2.4.1. Talent checking and confirming that shutter 2 is closed.

2.4.2. Talent using a pipette or dropper to apply immersion media onto the microscope objective.

2.4.3. Talent positioning the external detector on the microscope stage and aligning it with the objective lens.

2.5. Now turn on the scanning galvanometer mirrors to set their voltage to zero **[1]**. Open the shutter **[2]** and use a fluorescent target to confirm that the beam reaches the external detector **[3]**.

2.5.1. Talent switching on the galvanometer controller.

2.5.2. Talent opening the shutter.

2.5.3. Talent verifying the laser beam path using a fluorescent target.

2.6. Set the autocorrelator detector to be the external detector **[1]**. Then adjust the stage position until the signal appears **[2]**.

2.6.1. SCREEN: 68637_screenshot_2.mp4. 00:00-00:10

2.6.2. Talent gently adjusting the stage position while monitoring the signal.

2.7. Adjust the polynomial coefficients of the pulse shaper to iteratively minimize the pulse width measured by the autocorrelator **[1]**. As the pulse width decreases, reduce the scan range accordingly to improve the accuracy and visualization of the autocorrelation function **[2]**.

2.7.1. SCREEN: 68637_screenshot_3.mp4 00:09-00:30. **AND** 68637_screenshot_4.mp4. 00:27-00:42
Video Editor: Please play both shots side by side

2.7.2. SCREEN: 68637_screenshot_5.mp4. 00:00-00:23

3. SLAM Microscope Initialization, Imaging, and Shutdown Procedure

3.1. Ensure that the laser input to the photonic crystal fiber is blocked and turn on the laser **[1]**. Allow the laser to warm up for 30 minutes **[2]**.

3.1.1. WIDE: Talent confirming the beam path is blocked at the PCF input and powering on the laser.

3.1.2. Shot of a 30-minute timer being started on a phone.

3.2. Verify that the beam path is free of obstructions **[1]**. Then place an optical power meter in the beam path just before the photonic crystal fiber **[2]**. Press the control button to open shutter 1 **[3]** and measure the input power **[4]**.

3.2.1. Talent scanning and checking the entire beam path for any obstacles.

3.2.2. Talent placing the power meter before the PCF input.

3.2.3. Talent opening shutter 1 by pressing electronic control button.

3.2.4. Talent looking at power values from the meter.

3.3. Close shutter 1 again **[1]**. Move the power meter to the output of the photonic crystal fiber after the parabolic mirror **[2]**. Open shutter 1 **[3]** and use the input 3-axis stage to maximize the output power **[4]**.

3.3.1. Talent blocking the laser at the source.

3.3.2. Talent positioning the power meter at the PCF output, after the parabolic mirror.

3.3.3. Talent opening shutter 1 by pressing electronic control button.

3.3.4. Talent adjusting the knobs of the 3-axis stage to maximize power, while

watching the meter.

3.4. Let the photonic crystal fiber warm up for 10 minutes, then maximize the output power again using the 3-axis stage **[1]** and record the transmission of the PCF **[2-TXT]**.

3.4.1. Talent fine-tuning the 3-axis stage to maximize power output again.

3.4.2. Talent recording PCF transmission value in the fiber log spreadsheet on the computer. **TXT: IF the transmission falls below 50%, replace the PCF**

3.5. Move the power meter after the neutral density filter **[1]** and rotate the filter to adjust the laser power reaching the sample **[2]**. Multiply the measured power by the transmittance of the remaining optics to determine sample power **[3]**.

3.5.1. Talent placing the power meter downstream of the neutral density filter.

3.5.2. Talent rotating the neutral density filter dial while observing power readings.

3.5.3. SCREEN: 68637_screenshot_6.mp4. 00:00-00:11

3.6. Now, turn on the galvanometers and stage **[1]**. Launch the acquisition software and enter the desired imaging parameters **[2]**.

3.6.1. Talent switching on the galvanometer and stage power supplies. NOTE: 3.6.1 is filmed in 2 takes

3.6.2. SCREEN: 68637_screenshot_7.mp4. 00:00-00:11

3.7. Place the sample on the stage using the same immersion media that was applied earlier to the objective lens **[1]**. Then turn off the room lights **[2]** and close the curtains to prevent ambient light from entering **[3]**.

3.7.1. Talent applying immersion media and placing a prepared sample slide onto the microscope stage.

3.7.2. Talent flipping the room light switch off.

3.7.3. Talent pulling curtains shut around the microscope.

3.8. Next, switch on the detector power supplies and amplifiers **[1]**. Shut the light box surrounding the microscope stage **[2]**. Use the electronic control switches to manually open the detector shutters 3 to 6 **[3]**.

3.8.1. Talent pressing the switches to turn on the detectors and associated amplifiers.

3.8.2. Talent closing the shielding enclosure or light-tight box around the microscope stage.

3.8.3. Talent pressing electronic toggle switches labeled S3, S4, S5, and S6 to open shutters.

3.9. In the acquisition software, press the **Click to Start Acquisition** option to begin SLAM *(Slam)* imaging **[1]**. Adjust the microscope focus using the stage controller knob until the sample is clearly visible **[2]**.

3.9.1. SCREEN: 68637_screenshot_7.mp4 00:12-00:30

3.9.2. SCREEN: 68637_screenshot_7.mp4 00:30-00:50

3.10. Use the joystick on the microscope stage controller to find the desired field of view **[1]**. Once positioned, click **Stop and Reset [2-TXT]**.

3.10.1. Talent using joystick to navigate within the sample area.
AND
SCOPE/SCREEN: 68637_screenshot_7.mp4 01:40-01:59
Video Editor: Please play both shots side by side

3.10.2. SCREEN: 68637_screenshot_7.mp4 01:59-02:02
TXT: If image saturates, repeat process from laser power control

3.11. Under the **Saving** tab, toggle **Save Data** to **Yes [1]**, then press on the **Click to Start Acquisition** option and wait for the imaging to complete **[2]**.

3.11.1. SCREEN: 68637_screenshot_7.mp4 02:22-02:34

3.11.2. SCREEN: 68637_screenshot_7.mp4 02:34-02:55

3.12. To manipulate the sample during imaging, close the detector shutters and use a flashlight or headlamp for illumination **[1]**. After adjustments, return to the imaging setup **[2]**.

3.12.1. Talent turning off detector shutters and using a headlamp while modifying the sample on the stage.

3.12.2. Talent returning to stage and reinitializing imaging setup.

3.13. To end image acquisition, close the detector shutters **[1]**, turn off the detectors **[2]**, and only then switch on the room lights **[3]**.

3.13.1. Talent pressing switches to close all detector shutters.

3.13.2. Talent powering down detector units.

3.13.3. Talent turning on room lights.

3.14. Block the laser using shutter 1 **[1]**, then turn off the laser **[2]**. Turn off the galvanometers and stage **[3]** and close the acquisition software **[4]**.

3.14.1. Talent pressing the electronic control button of the shutter to block the beam.

3.14.2. Talent switching off the laser unit.

3.14.3. Talent powering down galvanometers and stage controller.

3.14.4. SCREEN: SCREEN: 68637_screenshot_7.mp4 04:03-04:10

3.15. Dispose of the tissue sample appropriately and clean the workstation **[1]**.

3.15.1. Talent discarding used tissue in biohazard bin and wiping down the microscope stage with disinfectant.

Results

4. Results

4.1. A properly cleaved photonic crystal fiber showed a flat end face perpendicular to its axis **[1]** and a clean face **[2]**.

4.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight image A*

4.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight image C*

4.2. Poorly cleaved photonic crystal fibers showed angled cuts, chipped faces **[1]**, and pronounced scratches or debris on the surface **[2]**. Improper handling or contamination resulted in visible dirt and particulate buildup around the fiber surface, obscuring the air hole pattern **[3]**. A burned core with darkened regions indicated a damaged photonic crystal fiber at the end of its life **[4]**.

4.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight image B*

4.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight image E*

4.2.3. LAB MEDIA: Figure 2 *Video editor: Highlight image D*

4.2.4. LAB MEDIA: Figure 2 *Video editor: Highlight image F*

4.3. The supercontinuum spectrum spanned approximately 975 to 1175 nanometers with a flat top and distinct spectral modulations **[1]**. Pulse autocorrelation after compression showed a symmetrical profile with a narrow full-width half-maximum, indicating good pulse shaping **[2]**.

4.3.1. LAB MEDIA: Figure 3 *Video editor: Highlight image A*

4.3.2. LAB MEDIA: Figure 3 *Video editor: Highlight image B*.

4.4. Properly functioning supercontinuum sources produced strong second- and third-harmonic signals **[1]**. A narrowed supercontinuum resulted in loss of third-harmonic signal intensity while retaining the second-harmonic signal **[2]**. Absence of pulse shaping drastically reduced all nonlinear signals, yielding a faint and low-contrast image **[3]**.

4.4.1. LAB MEDIA: Figure 4. *Video editor: Sequentially highlight the dense green and magenta coloration in image A*

4.4.2. LAB MEDIA: Figure 4. *Video editor: Highlight the image B*

4.4.3. LAB MEDIA: Figure 4. *Video editor: Highlight the image C*

4.5. Successful SLAM imaging of *ex vivo* kidney tissue showed distinct nephron structures with multiple strong optical signals including third-harmonic generation and NADPH fluorescence **[1]**. Renal interstitial collagen fibers were identifiable via strong second-harmonic generation signals **[2]**.

4.5.1. LAB MEDIA: Figure 5 *Video editor: Highlight the THG (magenta) and NAD(P)H (Cyan) signals in images A and C*

4.5.2. LAB MEDIA: Figure 5. *Video editor: Highlight the SHG (Green) signals in images A and C*

4.6. The redox ratio image indicated elevated aerobic mitochondrial metabolism in the proximal tubules of the renal cortex compared to the medulla **[1]**. A second redox ratio map confirmed spatial variation in oxidative and glycolytic metabolism, reinforcing differential energy usage within the tissue **[2]**.

4.6.1. LAB MEDIA: Figure 5B.

4.6.2. LAB MEDIA: Figure 5D.

Pronunciation Guide

1. **Supercontinuum**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/supercontinuum
   - **IPA (American)**: /ˌsuːpərkənˈtɪnjuəm/
   - **Phonetic Spelling**: soo-per-kuhn-TIN-yoo-uhm
2. **Photonic**
   - **Pronunciation link**: https://www.merriam-webster.com/medical/photonic
   - **IPA (American)**: /foʊ-ˈtän-ik/ (fotonic) Cambridge DictionaryMerriam-Webster+6Merriam-Webster+6Merriam-Webster+6
   - **Phonetic Spelling**: foh-TAW-nik
3. **Galvanometer**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/galvanometer
   - **IPA (American)**: /ˌgal-və-ˈnä-mə-tər/ (gal-və-NAH-mə-ter) YouTube+10Merriam-Webster+10Oxford English Dictionary+10
   - **Phonetic Spelling**: gal-və-NAH-mə-ter
4. **Dispersion**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/dispersion
   - **IPA (American)**: /dɪ-ˈspɝː-ʒən/ (di-SPUR-zhun) YouTube+15Cambridge Dictionary+15Cambridge Dictionary+15Cambridge Dictionary+15Merriam-Webster+15How To Pronounce+15

- **Phonetic Spelling**: di-SPUR-zhun

5. **Immersion**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/immersion
   - **IPA (American)**: /ɪ-ˈmɝː-ʒən/ (ih-MUR-zhun) Merriam-Webster
   - **Phonetic Spelling**: ih-MUR-zhun
6. **Autocorrelator** *(assuming from autocorrelation)*
   - **Pronunciation link**: While Merriam-Webster has "autocorrelation," an "autocorrelator" is commonly derived. Unfortunately, no direct page was found—so: "No confirmed link found."
   - **IPA (American)**: /ˌɔːtəʊ-ˈkɒrə-leɪtər/
   - **Phonetic Spelling**: aw-TOH-KOR-uh-lay-tur
     (Note: This is a linguistically standard derivation, though not sourced.)
7. **Photonics**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/photonics
   - **IPA (American)**: /foʊ-ˈtä-niks/ (fo-TAW-niks) YouTube+15Forvo.com+15How To Pronounce+15Merriam-WebsterMerriam-WebsterMerriam-Webster+1
   - **Phonetic Spelling**: fo-TAW-niks
8. **Phonetic**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/phonetic
   - **IPA (American)**: /fə-ˈnet-ik/ (fə-NET-ik) Merriam-Webster+1
   - **Phonetic Spelling**: fə-NET-ik
9. **Phonetics**
   - **Pronunciation link**: https://www.merriam-webster.com/dictionary/phonetics
   - **IPA (American)**: /fə-ˈne-tiks/ (fə-NET-iks) Merriam-Webster
   - **Phonetic Spelling**: fə-NET-iks
10. **Immerse** *(related to immersion)*
    - **Pronunciation link**: https://www.merriam-webster.com/dictionary/immerse
    - **IPA (American)**: /ɪ-ˈmərs/ (ih-MURS) Encyclopedia Britannica+12Merriam-Webster+12Merriam-Webster+12
    - **Phonetic Spelling**: ih-MURS