Grant name: SISGR: Room Temperature Single-Molecule Detection and Imaging by Stimulated Emission Microscopy

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Executive Summary:

1. How the research adds to the understanding of the area investigated

This research aims to push the detection sensitivity of stimulated Raman scattering (SRS) imaging to single molecule. In the past, single molecule studies rely on fluorescent labeling, which is not always possible. We explored several techniques to address the difficulties in detecting single molecule with SRS: By using near-resonance SRS with visible lasers we achieved a 10-molecule detection sensitivity. We developed frequency-modulation spectral-focusing (FMSF) SRS and multi-color SRS to decrease imaging background and molecule bleaching. We also tested other methods in order to overcome the shot noise that limit the sensitivity of SRS. These methods have provided important imaging improvements for the visualization of molecules in low concentration with SRS.

2. The technical effectiveness and economic feasibility of the methods or techniques investigated or demonstrated

The near-resonance SRS uses the very similar setup as conventional SRS setup. Second harmonic generation (SHG) crystals and focusing lenses are added to the setup to generate SHGs from both pump and Stokes laser. These modifications are easily implemented and do not add much cost to the conventional SRS setup. FMSF-SRS's laser costs about the same as conventional picosecond OPO systems. It uses glass rods to chirp the femtosecond pulses to picosecond. The multi-color SRS is achieved by adding simple optical components or fiber amplifier to the system. Therefore, the newly developed methods do not add a significant extra cost to the existing conventional SRS system and thus are economically feasible.

3. How the project is otherwise of benefit to the public

The method developed for this project is generally applicable to label-free chemical imaging of any molecules in low concentration. Because nonlinear optical spectroscopy and microscopy are such a valuable tool for studying chemical reactions as well as biomedical sciences, we believe this project will benefit a variety of scientific researches which will deepen our understanding of energy production processes and human diseases. As an example, we are the

first to be able to image neurotransmitter in live tissue. We have also demonstrated the imaging of white blood cells in blood stream in live animals using simultaneous two-color SRS.

Comparison: compare actual accomplishments with goals and objectives of the project

Original Project Objectives

The objective of this proposal was to push the sensitivity of stimulated Raman scattering microscopy to a single molecule limit in order to probe chemical and biochemical reactions. By pursuing the following specific aims:

1) Detect and image single molecules based on their Raman transitions. Pursued by using a near-resonance stimulated Raman scattering (SRS) microscopy system. A dramatic improvement in the ability to detect strong Raman transitions of single molecules such as β-carotene will push the sensitivity limit of SRS microscopy, so that it compliments similar single-molecule techniques based on fluorescence and absorption.

2) Demonstrate key applications of monitoring the chemical reactions of single nonfluorescent molecules:

● Using near-resonance SRS as a contrast mechanism to study dye-sensitize semiconductor interface, elucidating the heterogeneous electron ejection kinetics with high spatial and temporal resolution.

● Monitoring electron transfer and proton translocation reactions catalyzed by a single cytochrome c oxidase undergoing continuous enzymatic turnovers

● Determining the mechanism of cooperative ligand binding of hemoglobin among its four subunits

Actual Accomplishments

Over the last funding period our group has several technical developments on stimulated Raman scattering (SRS) microscopy, which allow us to push the sensitivity of label free chemical imaging to an unprecedented level. Exciting new applications have been enabled by these new techniques that do not rely on fluorescence. Specifically, we made significant progress in three new technologies: near-resonance SRS, frequency modulation spectral focusing SRS, multi-color SRS, and other time domain coherent Raman methods.

1. Near-resonance SRS microscopy

Raman scattering cross section depends greatly on the excitation wavelength. Near-resonance SRS uses visible lasers and has the advantage to boost Raman signals without exciting molecules to the electronical excitation state. With this method, we are able to push the sensitivity to the level of 10 molecules in laser focus. However, multi-photon excitation can still damage molecules before enough signals are collected. To overcome this problem, we successfully synthesized a beta-carotene molecule covalently conjugated to a triplet state quencher molecule cyclooctatetraene. However, compared with beta-carotene, the synthesized

molecule does not show improved photostability when excited with 532nm and 578 nm picosecond lasers.

2. Frequency modulation spectral focusing SRS microscopy

To confirm a signal is from a single molecule, it is necessary to take on- and off-resonance images and subtract them to remove background. Although SRS imaging has a major advantage compared to CARS in that it automatically removes non-resonant four-wave mixing background, it is still susceptible to cross-phase modulation (XPM) induced background, especially when the SRS signal is extremely weak. Due to molecular instability, such subtraction is necessary to be performed in real time. Based on a previously demonstrated femtosecond spectral focusing SRS technique (Fu, Holtom, Freudiger, Zhang, & Xie, 2013), we have successfully demonstrated frequency-modulation SRS by rapidly switching the time delay between the pump and probe pulses at 20 MHz. Because in spectral focusing SRS, the probed Raman frequency dependence is linear on the time delay, XPM-induced background is effectively removed in real-time by up to 10 folds. With this setup we could demonstrate the application of this sensitive chemical imaging technique in biology by imaging the neurotransmitter acetylcholine in live tissue for the first time as shown in Figure 1 (Fu, Yang, & Xie, 2016).

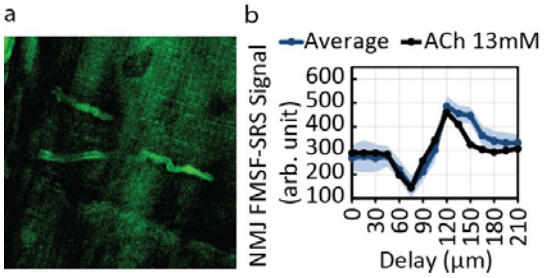

Figure 1. a. Acetylcholine in frog neuromuscular junction visualized by frequency-modulation spectral-focusing SRS. b. Estimation of concentration of acetylcholine in the tissue.

3. Multi-color SRS microscopy

Compared with frequency modulation, multi-color SRS is one step further for chemical imaging. To further reduce the background, it is necessary to perform more complex spectral analysis than just the subtraction we discussed above. We developed a pico-femto multi-color SRS microscopy to simultaneously probe four colors (Lu et al., 2012). We developed a spectral phasor method to analyze the hyperspectral imaging data (Fu & Xie, 2014). We have also developed a simultaneous two-color (STC) SRS setup by adding a simple fiber amplifier to a standard OPO laser source (Yang, Li, Suo, Lu, & Xie, 2017). The great advantage of the STC-SRS is that it allows for epi-detection and easy scalable to multi-colors. To validate our method we demonstrated a biomedical application of STC-SRS by successfully imaging white blood cells in blood streams of live mice as shown in Figure 2.

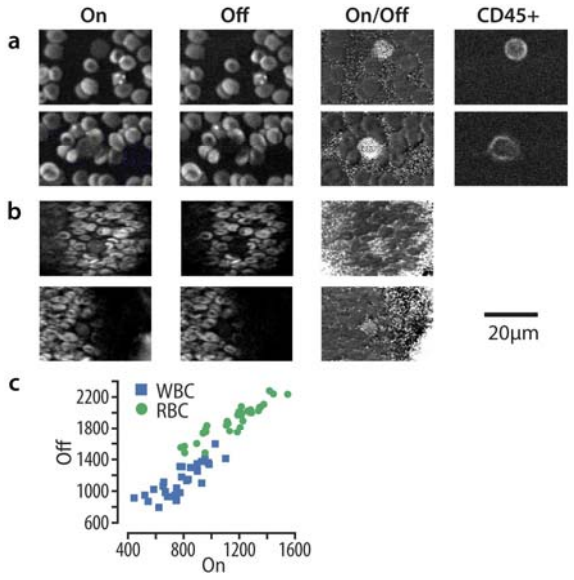

Figure 2. a. *Ex vivo* imaging of white blood cells among red blood cells with STC-SRS. b. *In vivo* imaging of white blood cells in blood stream with STC-SRS. c. the distribution of signals of two SRS channels from white and red blood cells.

4. Time-domain coherent Raman microscopy

To improve the sensitivity of coherent Raman microscopy, we aimed at reducing the background noise. We tried time-resolved femtosecond CARS with frequency modulation to remove the non-resonant background. We achieved sensitivity at the same level as conventional SRS, primarily because CARS signal depends on chemical concentration quadratically, where the signal reduces proportionally to how fast the concentration of chemicals scales down. We also tested time gating SRS to perform time domain measurement of stimulated Raman gain. However, without the heterodyne signal amplification, the time-domain with SRS doesn't have high enough sensitivity.

Summarize project activities for the entire funding period:

In the last proposal period, we have successfully pushed the detection limit of label-free stimulated Raman scattering microscopy to the level of 10 molecules. SRS is advantageous to previous absorption based pump-probe methods because of its high chemical specificity, which allows to target the molecules at lower concentration, important in biology and medical applications. The new methods developed in the fund period of this grant have advanced the detection sensitivity in many aspects. Near-resonance SRS improved the signal by using shorter wavelengths for SRS microscopy. Frequency modulation and multi-color SRS target the reduction of background to improve the chemical specificity of SRS while maintaining the high imaging speed. Time-domain coherent Raman scattering microscopy targets to reduce the noise floor of coherent Raman microscopy. These methods have already demonstrated first-of-a-kind new applications in biology and medical research. However, we are still one order of magnitude away from single molecule limit. It is important to continue to improve the laser

specification and develop new imaging methods to finally achieve label-free single molecule microscopy.

Products: List publications, and other products

Publications of DOE/BES sponsored research

Fu, D., Holtom, G., Freudiger, C., Zhang, X., & Xie, X. S. (2013). Hyperspectral imaging with stimulated Raman scattering by chirped femtosecond lasers. *The Journal of Physical Chemistry B, 117*(16), 4634-4640.

Fu, D., & Xie, X. S. (2014). Reliable cell segmentation based on spectral phasor analysis of hyperspectral stimulated Raman scattering imaging data. *Analytical Chemistry, 86*(9), 4115-4119.

Fu, D., Yang, W., & Xie, X. S. (2016). Label-free Imaging of Neurotransmitter Acetylcholine at Neuromuscular Junctions with Stimulated Raman Scattering. *Journal of the American Chemical Society*.

Lu, F.-K., Ji, M., Fu, D., Ni, X., Freudiger, C. W., Holtom, G., & Xie, X. S. (2012). Multicolor stimulated Raman scattering microscopy. *Molecular Physics, 110*(15-16), 1927-1932.

Yang, W., Li, A., Suo, Y., Lu, F.-K., & Xie, X. S. (2017). Simultaneous two-color stimulated Raman scattering microscopy by adding a fiber amplifier to a 2 ps OPO-based SRS microscope. *Optics letters, 42*(3), 523-526.