Monitoring Lipid Accumulation in the Green Microalga *Botryococcus braunii* with Frequency-Modulated Stimulated Raman Scattering

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ABSTRACT

The potential of microalgae as a source of renewable energy has received considerable interest because they can produce lipids (fatty acids and isoprenoids) that can be readily converted into biofuels. However, significant research in this area is required to increase yields to make this a viable renewable source of energy. An analytical tool that could provide quantitative in situ spectroscopic analysis of lipids synthesis in individual microalgae would significantly enhance our capability to understand the synthesis process at the cellular level and lead to the development of strategies for increasing yield. Stimulated Raman scattering (SRS) microscopy has great potential in this area however, the pump-probe signal from two-color two-photon absorption of pigments (chlorophyll and carotenoids) overwhelm the SRS signal and prevent its application. Clearly, the development of a background suppression technique is of significant value for this important research area.

To overcome the limitation of SRS in pigmented specimens, we establish a frequency-modulated stimulated Raman scattering (FM-SRS) microscopy that eliminates the non-Raman background by rapidly toggling on-and-off the targeted Raman resonance. Moreover, we perform the background-free imaging and analysis of intracellular lipid droplets and extracellular hydrocarbons in a green microalga with FM-SRS microscopy. We believe that FM-SRS microscopy demonstrates the potential for many applications in pigmented cells and provides the opportunity for improved selective visualization of the chemical composition of algae and plants

Keywords: stimulated Raman scattering, microscopy, frequency modulation, lipids, hydrocarbon, biofuels, microalgae, *Botyrococcus braunii*

1. INTRODUCTION

Recently, the colonial green microalga, known as *Botryococcus braunii*, has attracted great attention because it produces large quantities of hydrocarbons that can be readily converted into biofuels [1-3]. However, there is a need for improved label-free, non-invasive quantitative microanalysis of intracellular lipid droplets and extracellular hydrocarbons with subcellular resolution. The excellent chemical specific and label-free capabilities of coherent Raman techniques including coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) [4] allow scientists to image and analyze different microalgae [5-8] and plants [9-14] in the past decade. However, due to interference from other pump-probe interactions such as two-color two-photon absorption (TPA) and photothermal lensing effects [15-17] in pigments such as chlorophyll and carotenoids, the amplitude-modulated stimulated Raman scattering (AM-SRS) microscopy is severely restricted for *in vivo* imaging in these samples. In most samples the TPA is a weak effect, however heavily pigmented samples, such as *Botryococcus braunii,* the optical absorption is significantly strong across a broad spectral range and TPA overwhelms the SRS signal. Clearly, the development of detections techniques that isolate the vibrationally resonant SRS signal from broad-band electronic absorption are vital in order to apply SRS imaging in this important research area..

Multiphoton Microscopy in the Biomedical Sciences XV, edited by Ammasi Periasamy, Peter T. C. So, Karsten König,
Proc. of SPIE Vol. 9329, 93291J · © 2015 SPIE · CCC code: 1605-7422/15/$18 · doi: 10.1117/12.2077835

To this end we demonstrate that frequency-modulated stimulated Raman scattering (FM-SRS) microscopy of *Botryococcus braunii* eliminates the non-Raman background by rapidly toggling on-and-off the targeted Raman resonance [15, 17, and 18]. Moreover, we show background-free imaging and hyperspectral analysis of intracellular lipid droplets in the green microalgae with FM-SRS microscopy.

2. MATERIALS AND METHODS

Frequency Modulation

The light sources used for FM-SRS imaging comprised of a Nd:Vanadium pico-second oscillator (picoTrain, HighQ laser) at 1064 nm and Ti:Sapphire laser (Mira 900, Coherent). The repetition rate of Ti:Sapphire laser was electronically syncronised to the Nd:Vanadium pico-second oscillator (Synchrolock, Coherent). To perform FM-SRS microscopy, a pulse shaper consisting of an acoustic optical modulator (AOMO 3080−122, Crystal Technology) and diffraction grating (1200 l/mm, Wasatch Photonics) were constructed to filter the broadband beam into a narrowband beam with a center wavelength that could be modulated in the MHz range. Filtering was achieved by placing an adjustable slit in front of the spectrally dispersed beam reflected from the grating. By using the AOM to rapidly scan the angle of incidence upon the grating allowed the pump beam toggling between 816.4 nm (2850.4 cm^{-1}, CH_2 stretch) and 821 nm (2781.8 cm^{-1}, off-resonance) at 1.7 MHz. The pump and Stokes beams were collimated on separate telescopes and then combined at an 850 nm short pass dichroic filter. A schematic of the optical setup is shown in Fig. 1.

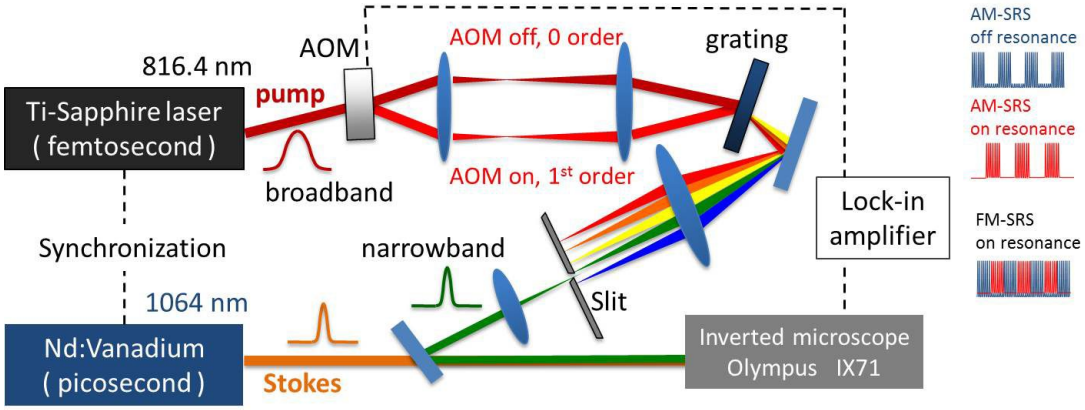

Figure 1: The optical setup of frequency-modulated stimulated Raman scattering microscopy.

Microscopy

All images were acquired by a modified confocal laser scan unit (Flouview 300, Olympus) and an inverted microscope (IX71, Olympus). The light was focused onto the sample using a water immersion 60× NA1.2 objective (UPlanSApo, Olympus) and collected by a water immersion 60× NA1.0 objective (LUMPlanFLN, Olympus). CARS and 2PEF images were detected in the epi-direction using a 750 nm long pass dichroic (750dcxr, Chroma) and two filters centered at 660 nm (660.0 IF 40D, Ealing Inc.) to separate the signal from the laser excitation. The forward propagated light was simultaneously collected using a 60× 1.0NA water immersion microscope objective for *Botryococcus braunii* samples. For stimulated Raman gain (SRG) imaging, the pump beam was blocked from the photodiode by an 850 nm long pass filter (hq850lp) and the Stokes beam was detected by a InGaAs switchable gain detector (PDA1010CS, thorlabs). To suppress the strong signal due to the laser pulsing at 76 MHz, the output current was filtered by a low pass filter (Mini-Circuits, BLP-1.9+) and then terminated by a 50Ω resistor. The photodiode was connected to a radio frequency lock-in amplifier (SR844, Stanford Research Systems) which detected the modulations in the at the 1.7 MHz frequency. The images were acquired at a size of 512x512 pixels with 53s frame rate. Image processing and analysis were performed by ImageJ (NIH open source software).

Sample preparation

Botryococcus braunii Guadeloupe (Race B) strain was obtained from Pierre Metzger (Laboratoire de Chimie Bioorganique et Organique Physique, Ecole Nationale Supérieure de Chimie de Paris, France). Cultures were grown in 1 L Erlenmeyer flasks using modified Chu-13 medium, and were incubated at 23ºC, continuous aeration with 5% atmospheric CO_2 enriched air, orbital shaking at 90 rpm and 18h light : 6 h dark photoperiod with a light flux density of 50 E $m^{-1}s^{-1}$. Two weeks grown cultures were removed from the flask, mounted whole between two coverslips and imaged immediately.

3. RESULTS

To validate the performance of the frequency modulation technique, we compared AM-SRS with FM-SRS on-resonance (2850 cm^{-1}) imaging of *Botryococcus braunii* samples. Fig. 2(A) and (B) show the AM-SRS imaging of the colonial green microalga with different sensitivities (30 µVrms and 300 µVrms) of lock-in amplifier. We notice that AM-SRS imaging achieve the saturation of detection in Fig. 2(A), whic originates from two-color two-photon absorption (TPA) in pigmented regions of the cells containing chlorophyll and carotenoids. In most biological specimens TPA is a weak effect, but in the case of *Botryococcus braunii* imaging TPA is significantly strong across a broad spectral range that TPA overwhelms the SRS signal. In contrast, the FM-SRS technique successfully eliminates the non-Raman background (Fig. 2(C)) and reveals the lipid droplets distribution.

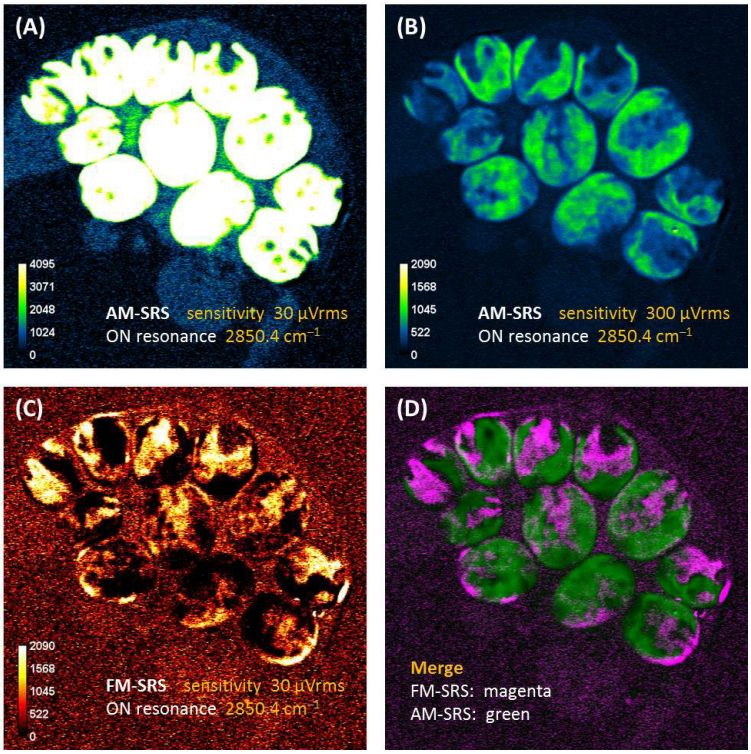

Figure 2: AM-SRS and FM-SRS imaging of *Botryococcus braunii*. Comparison of (A) AM-SRS and (C) FM-SRS images of *Botryococcus braunii* with CH2 stretch band (2850 cm-1). The AM-SRS image is dominated by TPA (B), whereas lipid droplets can be revealed by FM-SRS image (D). Field of view: 51.2 µm x 51.2 µm.

Since the frequency-modulation detection technique is of capability to perform background-free imaging [15], we further demonstrated the hyperspectral FM-SRS vibrational imaging of microalgae. The angle of the beam diffracted by the AOM was controlled by the driving frequency of the RF signal which was generated by a lock-in amplifier allowing the RF frequency to be adjusted manually. Generating a square-wave toggled the output of the filter between two wavelengths where the RF frequency controlled the diffraction angle of the first-order beam. Scanning the driving frequency allowed spectral acquisition of FM-SRS imaging of intracellular lipid droplets in *Botryococcu braunii* samples, scanning the CH_2 stretch band from 2820 cm^{-1} to 2860 cm^{-1} (Fig. 3(B) - (D)). The power of the pump beam was normalized against the spectral profile of the broadband pulses.

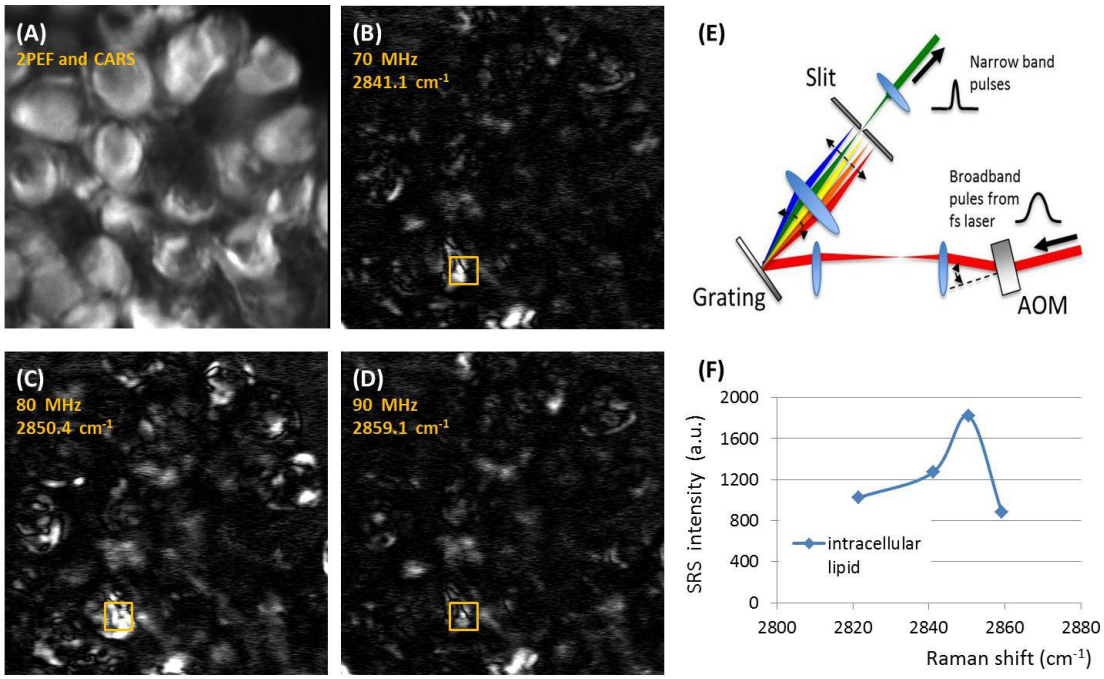

Figure 3: Hyperspectral FM-SRS imaging of *Botryococcus braunii*. (A) 2PEF and epi-CARS image. (B-D) Hyperspectral FM-SRS imaging of intracellular lipid droplets in microalgae was performed using different AOM driving frequency. (E) Schematic of the pulse shaper and AOM. (F) Spectral profile of CH_2 stretch modes. Field of view of (A-D): 42.67 μm x 42.67 μm.

4. CONCLUSION

This research presents our preliminary results regarding the technical issues associated with performing background-free imaging of *Botryococcus braunii* with FM-SRS microscopy. Our experimental results show that FM-SRS technique can successfully overcome the limitation of SRS in pigmented specimens and demonstrate the feasibility of hyperspectral vibrational imaging. We believe that FM-SRS microscopy is an ideal tool for imaging microalgae and worthy of further *in vivo* applications in plant physiology. Moreover the angle dependence of the AOM on driving frequency allows an elegant solution for rapid wavenumber tuning for hyperspectral imaging.

ACKNOWLEDGMENTS

We thank Delong Zhang at Purdue University for fruitful discussion. This research was supported by grants (BB/K013602/1) from the Biotechnology and Biological Sciences Research Council (BBSRC) and Syngenta.

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