Optical Trapping and Coherent Anti-Stokes Raman Scattering (CARS) Spectroscopy of Submicron-Size Particles

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Abstract—Optical trapping combined with coherent anti-Stokes Raman scattering (CARS) spectroscopy is demonstrated for the first time as a new technique for the chemical analysis of individual particles over an extended period of time with high temporal resolution. Single submicron-size particles suspended in aqueous media are optically trapped and immobilized using two tightly focused collinear laser beams from two pulsed Ti:Sapphire laser sources. The particles can remain stably trapped at the focus for many tens of minutes. The same lasers generate a CARS vibrational signal from the molecular bonds in the trapped particle when the laser frequencies are tuned to a vibrational mode of interest, providing chemical information about the sample. The technique is characterized using single polystyrene beads and unilamellar phospholipid vesicles as test samples and can be extended to the study of living biological samples. This novel method could potentially be used to monitor rapid dynamics of biological processes in single particles on short time scales that cannot be achieved by using other vibrational spectroscopic techniques.

Index Terms—Biophotonics, coherent anti-Stokes Raman scattering (CARS), laser tweezers, Raman spectroscopy.

I. INTRODUCTION

COHERENT anti-Stokes Raman scattering (CARS) microscopy has been developed recently [1]–[4] as a technique capable of high spatial resolution for the chemical imaging of live biological samples without the need for intrusive labeling with fluorescent probes. Contrast in CARS imaging is generated from vibrational signals of Raman active molecular vibrations in the sample itself. Consequently, a major benefit of CARS imaging is that the signal, unlike the signal from fluorescent probes, does not photobleach, enabling long-term imaging of a living sample as it undergoes biological processes. The coherent nature of the nonlinear CARS process results in vibrational signals that are much stronger than spontaneous Raman signals. Spontaneous Raman spectroscopy is known for its relatively low scattering cross-sections ($\sim 10^{-30}$ cm$^2$) that limit its practical use for imaging applications. The orders of magnitude higher signals in CARS microscopy allow for rapid CARS imaging approaching video rate speed over long time periods, which enables monitoring of rapid dynamic processes [5], [6] that are otherwise not feasible with conventional fluorescence or Raman microscopy.

In the CARS technique, two laser beams at two different frequencies ($\omega_p$, the pump frequency and $\omega_s$, the Stokes frequency) are tightly focused into the sample to generate a coherent anti-Stokes signal at frequency $2\omega_p - \omega_s$. Strong resonant CARS signals are generated when the frequency difference $\omega_p - \omega_s$ matches a Raman-active molecular vibration in the sample, which induces collective oscillations of the molecular bonds. Because CARS is a nonlinear multiphoton process, the signal is only generated within the focal volume, providing high spatial resolution akin to other multiphoton techniques, such as two-photon fluorescence and second harmonic generation. A CARS image is generated by rapid scanning of the laser focus over the entire biological sample.

An additional benefit of CARS microscopy is that sample autofluorescence does not usually interfere with the CARS signal, which is located at wavelengths shorter than the excitation wavelengths.

The long-term CARS analysis of submicron-size biological particles suspended in aqueous media is difficult, if not impossible, due to the constant Brownian motion of the particle in and out of the laser focus. Optical tweezers [7]–[11] have been shown to be able to immobilize a particle in solution such that it remains within the focal volume of the focused laser beam, essentially "trapping" the particle indefinitely. This trapping is enabled by photons from the laser beam imparting their momentum to the particle, which results in transverse and axial forces that are balanced near the laser focus. Several more recent studies [12]–[18] have already demonstrated the ability to combine optical trapping with spontaneous Raman spectroscopy using continuous wave (CW) laser sources, which allows for the chemical analysis of biological specimens and their dynamics.

However, this laser tweezers Raman spectroscopy (LTRS) technique still suffers from the inability to acquire spectra rapidly enough to monitor fast dynamics. Typically, spectra have to be acquired over periods of many seconds to minutes, thus allowing only slow, long-term dynamics (e.g., apoptosis) to be studied. CARS signals, in contrast, being that much stronger than spontaneous Raman signals, would enable monitoring of fast dynamics with higher temporal resolution and maintaining of chemical specificity.
Optical trapping with pulsed laser systems has not been as widely demonstrated as with CW lasers but has been gaining considerable attention due to the ability to study a trapped particle by nonlinear spectroscopic methods that require intense short laser pulses. Modeling studies [19] have predicted the feasibility of ultrashort-pulsed laser tweezers, and experimental studies have demonstrated the combination of trapping with SHG [20], [21] and two-photon fluorescence [22]. In this paper, we demonstrate the combination of optical trapping with CARS spectroscopy using two pulsed laser sources at different wavelengths that are overlapped both spatially and temporally. This technique makes it possible to rapidly obtain chemical information from submicron-size particles in their natural environment. The trapping parameters and CARS signal are demonstrated and fully characterized for polystyrene bead and unilamellar vesicle samples.

II. EXPERIMENTAL METHODS

A. Optical Raman and CARS Tweezers Setup

A schematic of the setup is shown in Fig. 1. Two 80-MHz 5-ps pulsed tunable Ti:Sapphire lasers (Tsunami, Spectra-Physics, Mountain View, CA) are temporally synchronized by phase-locked loop electronics (Lok-to-Clock, SpectraPhysics) and collinearly combined with a dichroic mirror. Both lasers are tunable from 750 nm ($1333.33 \text{ cm}^{-1}$) to 900 nm ($1111.11 \text{ cm}^{-1}$). The entire Raman spectral window from 500 to 2000 cm$^{-1}$ can be covered, which is the molecular fingerprint region of interest for biological samples where most information about cellular constituents (e.g., DNA, protein, lipids) can be found. Clean-up filters are used to suppress the amplified spontaneous emission from the Ti:Sapphire lasers within the spectral range where it can interfere with detection of the CARS signals. Laser power is controlled with polarization optics. The laser wavelengths are measured using a broadband fiber-optic handheld spectrometer (Ocean Optics, Dunedin, FL) with a resolution of roughly 1 nm. The parallel-polarized beams are expanded with a telescope assembly to approximately 5 mm and sent into an inverted optical microscope (Axiovert 200, Zeiss) equipped with a dichroic mirror and a 100 ×, 1.3 NA oil immersion objective (Plan-NEOFLUAR, Zeiss, Germany). CARS signals generated at the laser focus are epi-detected and sent through a 100-µm confocal pinhole and a short pass filter for rejection of the excitation light. The CARS signal is detected with either an avalanche photodiode or a grating spectrometer equipped with a liquid nitrogen cooled 1340 × 100 pixel charge-coupled device (CCD) camera (Roper Scientific, Trenton, NJ). The system is also equipped with a separate 30-mW He-Ne laser at 633 nm that is capable of trapping single particles and functions as an excitation laser for acquiring spontaneous Raman spectra. In addition, a pulse picker (NEOS Technologies, Melbourne, FL) can be added right after both beams are combined by the dichroic reflector to reduce the repetition rate of each laser beam down to 200 kHz. This reduces the average power of the beams and minimizes photodamage to samples, while maintaining high peak powers for CARS signal generation.

B. Preparation of Unilamellar Vesicles

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is purchased in powder form from Avanti Polar Lipids, Alabaster, AL. Liposomes are prepared using standard techniques [23]. Briefly, a DMPC/chloroform solution is dried under nitrogen for several minutes, placed under vacuum overnight to remove trace quantities of chloroform, and hydrated above its lipid transition temperature. Unilamellar vesicles are prepared through extrusion of the multimamellar vesicle solution using a mini-extruder (Avanti Polar Lipids). Approximately 1 mL of the hydrated lipid suspension is extruded several times at its phase transition temperature using polycarbonate membranes (Avanti Polar Lipids) with a 0.4-µm pore size to yield a concentrated solution of 0.4-µm diameter unilamellar vesicles. This solution is then diluted several times to a point where single vesicles can be observed and trapped at the laser focus.

III. TRAPPING OF POLYSTYRENE BEADS

A drop of solution containing 1-µm polystyrene beads (Duke Scientific Corp., Palo Alto, CA) is placed on a coverslip, which rests on a piezoelectric translation stage [Fig. 2(a)]. A single bead is stably trapped at the tight focus of the two collinearly combined laser beams, as shown in the white light image in Fig. 2(b), whereas other particles in the field of view are observed in constant motion and out of focus. It is known [2] that the intensity of epi-detected CARS signals is strongest for small scatterers when the diameter is on the order of the wavelength of the excitation light. Larger beads up to 4 µm can also be stably trapped. Typically, a single particle that is roughly 5 µm away from the laser focus will be drawn toward the laser focus due to the intensity gradient of the laser beam and become optically trapped. The particle is stably immobilized approximately 10 µm above the coverslip surface. Attempts to trap particles that are further away from the glass surface proves difficult, possibly
due to the distortion of the laser focus as it probes deeper into water. High laser powers (50 mW total) result in trapping for tens of seconds, at which point the particle will shoot out of the trap axially. It is not clear at this point whether this phenomenon is due to the instability of the trap at such high powers or laser damage to the bead, which results in potential bead deformation and increased absorption due to chemical modifications. When the power is reduced to 30 mW and below, the trap is much more stable and the bead remains immobilized for many minutes. Once trapped, the particle can be manipulated in the solution transverse to the beam by moving the translation stage and a few microns axially by moving the objective.

IV. CARS Characterization of Trapped Beads

Fig. 3(a) shows the intensity of the CARS signal when the two lasers are tuned on resonance to the 1001.5 cm\(^{-1}\) vibration of the polystyrene bead (\(\omega_p = 750\) nm, \(\omega_s = 811\) nm), which gives a CARS signal positioned at 697 nm. Typical pump and Stokes laser powers are 20 and 10 mW before the microscope objective and the spectra are acquired in 50 ms. No signal deterioration is observed, indicating that no bead modification or damage is occurring at these powers. In addition, the bead damage occurring at higher laser power is usually accompanied by an intense, broad fluorescence. We do not observe this fluorescence during the entire time the bead is maintained in the laser focus. For comparison, when the lasers are tuned off any polystyrene resonance vibration (e.g., at 900 cm\(^{-1}\)), a weaker nonresonant background signal that is inherent in CARS spectroscopy [4] is detected at 703 nm. The on-resonance signal is stronger by a factor of roughly five times the nonresonant background signal. No signal was obtained in the absence of a particle within the trap. The signals are confirmed to be CARS signals by determining the dependence of the signal on the pump and Stokes laser intensity, which is known to have a squared and linear dependence, respectively. Fig. 3(b) shows the log plots of the CARS intensity versus the pump and Stokes laser power and the slope values, which confirm the squared and linear dependence.

To confirm the stability of the trap and the signal from the particle, which is important if dynamic biological processes are to be inferred from small signal changes in the CARS signal intensity, the signal was acquired for many tens of seconds.
Fig. 3(c) shows the CARS signal from a trapped bead acquired for 10 s every 50 ms. The signal average is 600 counts with a standard deviation of 18 counts. On the seconds time scale, the signal is stable; however, signal fluctuations are observed in the millisecond regime. We attribute these fluctuations mainly to the timing jitter between the two laser beams that is inherent in the system. These fluctuations can be eliminated by using more sophisticated timing electronics to reduce this jitter, as has been demonstrated by Potma et al. [24] or by using other laser systems that do not rely on electronic locking of two independent laser systems, such as an optically parametric oscillator (OPO) system. It is possible that the fluctuations are also a result of variations of the bead position within the trap.

V. CARS TRAPPING WITH PULSE PICKED BEAMS

For CARS analysis of living biological samples, the use of high repetition rate and high average power pulsed lasers will result in damage to the sample. It has been demonstrated [2], [4] previously that incorporating pulse picking to reduce the average power of the laser beam while maintaining high peak pulse intensity, which is the important parameter for generating a CARS signal, can circumvent the issues of photodamaging biological samples. Here, we investigate the feasibility of maintaining stable optical trapping using lower powers with pulse picked laser beams and the simultaneous acquisition of CARS signals. A pulse picker is used to reduce the repetition rate of the laser beam to 4 MHz, 2 MHz, 800 kHz, and 400 kHz. At these repetition rates, the peak pulse energy can be maximized for CARS excitation (≈5 nJ) as opposed to ≈0.5 nJ for the data in the previous section), while maintaining low average powers of 20, 10, 4, and 2 mW, respectively. These powers are also sufficient for stable trapping and simultaneous CARS acquisition and are consistent with previous studies [20] showing that pulsed repetition rate lasers as low as 25 kHz can still optically trap nanometer-size particles as long as the frequency of the laser pulses surpasses the time it takes for the particle to move beyond its root mean square (rms) displacement from its equilibrium position.

To determine whether the CARS signal fluctuations at the millisecond time scale, such as those observed in Fig. 3(c), could be attributed to bead motion within the optical trap, we need to approximate the rms value of the particle position. It has been reported that 80-nm particles have an rms value of 20 nm for 40 μs, which corresponds to 25 kHz [20]. For the 1-μm particles and the 400 kHz–80 MHz laser repetition rates that are used in this study, the rms values would be even smaller (~1 nm and less). Therefore, we determine that the impact of the displacement of the 1-μm bead should not have a significant effect on the signal fluctuations.

It should be pointed out that at slightly lower powers than those specified previously, the intensity gradient of the laser beam still affects the natural Brownian motion of the particle, drawing it into the focus even though it does not remain stably trapped. This information is of particular interest for application of CARS toward correlation spectroscopy (CS). Cheng et al. [25] reported the use of CARS-CS to probe diffusion dynamics of nanometer-size polystyrene beads. For this application, particles are allowed to diffuse naturally into the focal volume of the CARS excitation beams, whereupon a CARS signal burst will be detected. Detection of these signal bursts and an autocorrelation analysis provides information about the diffusion dynamics of the particles. It should be noted that it is important that the laser beams not influence the natural diffusion of the beads in order for accurate dynamics to be recorded. In their study, laser beams with a repetition rate of 400 kHz were used at average powers slightly lower than the powers we reported above for trapping of particles. Their study also used smaller particles as well (175-nm particles). There is clearly a power level above which the laser forces influence the particle motion and below which there is no noticeable effect. Further studies are currently under way to investigate this dependence on laser power and particle size, shape, and optical properties, all of which are important for the CARS-CS technique.

VI. CARS TRAPPING OF UNILAMELLAR VESICLES

Previous studies [23], [26], [27] have used laser trapping with Raman spectroscopy to obtain the Raman vibrational fingerprint of unilamellar vesicles. CARS spectroscopy has also been applied to the study of lipid bilayers and liposomes on a substrate [28]–[31] Here, we demonstrate the optical trapping of unilamellar vesicles and the acquisition of CARS signals at a specific molecular vibration.

Single vesicles can be trapped stably (at 80 MHz) using as little as 10 mW total laser power. Because the vesicles are only 0.4 μm in diameter, often several vesicles will end up clustering into the laser focus. Typically, around five vesicles are observed to be clustering at one time, which can be avoided by diluting the vesicle solution appropriately. Fig. 4(a) shows the Raman spectrum from a single unilamellar DMPC vesicle trapped and excited using the 633-nm CW laser. The peaks are consistent with previously reported Raman spectra of DMPC vesicles. The 1440 cm⁻¹ peak, assigned to a CH₂ bending mode of the lipids, is chosen to generate the CARS signal and is wide enough to enable tuning of the lasers over the entire width of the peak [full-width at half-maximum (FWHM) 40 cm⁻¹] to trace out the profile of a CARS spectrum. Because we are currently limited by the resolution of the broadband spectrometer used to measure the laser wavelength, we are only able to collect five data points. Each data point is an average of many CARS signals collected from different trapped unilamellar vesicles. The laser power of the pump (750 nm) and Stokes (841 nm) laser beams are 30 mW and 15 mW, respectively. Fig. 4(b) shows the CARS spectral response (square points) overlaid on a magnified trace of the spontaneous Raman peak from Fig. 4(a), which mirrors the Raman vibrational profile of the 1440 cm⁻¹ peak.

VII. CONCLUSION

In conclusion, we have, for the first time, demonstrated the combination of optical tweezers with CARS spectroscopy. CARS signals from individual submicron-size objects such as polystyrene beads and unilamellar lipid vesicles were obtained with millisecond temporal resolution. CARS tweezers spectroscopy is a novel method that can potentially be used for
the label-free study of rapid exchange processes in micron-size biological particles (e.g., bacterial spores, liposomes, microbes). Although signal fluctuations on the millisecond time scale may be a major limitation for studying fast dynamics, we believe this novel method can be used to acquire information on the millisecond to second time scale after improvements are made to the system. This temporal resolution is greater than what spon-
tisecond to second time scale after improvements are made to be a major limitation for studying fast dynamics, we believe this

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ACKNOWLEDGMENT

The authors would like to thank M. Smith for the preparation of the multilamellar vesicle lipid suspension.

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