Characterization of Biological Structures with Nonlinear Chemical Imaging Nanomicroscopy

Richard D. Schaller, Justin C. Johnson, Kevin R. Wilson, Lynn F. Lee, Louis H. Haber, Richard J. Saykally (Department of Chemistry, University of California, Berkeley, CA 94720-1460)

1. Abstract

Techniques of coherent nonlinear spectroscopy (second harmonic generation and third harmonic generation) are combined with near-field scanning optical microscopy for imaging selected chemical and physical environments in biological matter on a nanoscopic scale. Resonant enhancement of nonlinear signals is utilized as a method of producing chemically selective contrast while the order of the process provides environmental selectivity. Systems studied include natural killer cells and erythrocytes.

2. Introduction

Nonlinear sample responses can be a useful in imaging for a variety of reasons. The first is that the magnitude of the nonlinear response of a sample can typically vary by orders of magnitude more than the linear response.\(^1-^3\) This is most clearly demonstrated in the case of linear absorption vs. nonlinear harmonic generation, where linear absorptions typically vary by ca. 10 percent in a sample whereas harmonic generation can increase in efficiency by $10^4$ with resonant enhancements.\(^1\) Nonlinear optical experiments can, in many instances, also constitute low background measurements in the same manner as fluorescence because the photons incident on the sample are not at the same frequency as those which are detected, thereby reducing background and increasing signal to noise ratios. Also, depending on the translational and molecular symmetry of a sample as well as the order of the nonlinear optical process, interfacial (anisotropic) or bulk (isotropic) sensitivity can be obtained within the dipole approximation.\(^1\)

3. Theory

Environmental selectivity of isotropic or anisotropic environments can be obtained in nonlinear imaging due to different selection rules for even- vs. odd-order processes.\(^1\) Chemically selective contrast is produced in NCIN imaging via the resonant component of the $n^{th}$ order susceptibility,

$$\chi^{(n)}_{\text{TOTAL}} = \chi^{(n)}_R + \chi^{(n)}_{NR},$$

where $\chi_{NR}$, the non-resonant part of the total susceptibility, is essentially wavelength independent and $\chi_R$ can be described, here for the more explicit example of SFG (SHG being the simplified case of SFG with degenerate photons), as\(^1\)
\[ \chi_R^{(2)} \approx \sum_{n,n' \neq g} \left( \frac{g[n']}{(\omega_{SF} - \omega_{ng} + i\Gamma_{ng})} \right) \times \left( \frac{<n|j|n'\rangle <n'|g\rangle}{(\omega_1 - \omega_{ng'} + i\Gamma_{ng'})} \right) + \frac{<n|k|n'\rangle <n'|g\rangle}{(\omega_2 - \omega_{ng'} + i\Gamma_{ng'})} \right). \]  

Here, when an allowed transition between states (ground, \( g \), and intermediate states, \( n \)) occurring at \( \omega_{ng} \) frequency becomes degenerate with the frequency of an input (\( \omega_1 \) or \( \omega_2 \) or \( |\omega_{SF}\rangle = |\omega_1 + \omega_2\rangle \) photon the denominator of a term approaches zero leaving only the imaginary lifetime of the transition (\( \Gamma^{-1} \)), resulting in a large increase (10^4) in efficiency of the nonlinear process being probed. Such resonant enhancement can effect chemically specific imaging due to the chemical character of the transition frequencies in the involved in the nonlinear process (e.g. vibrational or electronic).

The near-field detected nonlinear signal intensity (I), which is proportional to the square of the generated nonlinear induced polarization, for coherent nonlinear optical processes conducted in oblique collection mode can be expressed as the far-field signal intensity1,4,5 attenuated by near-field microscopy terms.6 In the case of SFG the expression is

\[ I(\omega_{SF}) = \frac{8\pi^3 (\omega_{SF})^2}{c^3 |\epsilon(\omega_{SF})\epsilon(\omega_1)\epsilon(\omega_2)|^2} \left| \chi_R^{(2)} : \epsilon_{\omega_{SF}} \epsilon_{\omega_{SF}} \right|^2 I(\omega_1) I(\omega_2) \left( \frac{r}{\lambda} \right)^6 \int_0^l e^{-(r+S)/\lambda} dS \right)^2, \]  

and for the case of THG is

\[ I(3\omega) = \frac{576\pi^4 \omega^2}{c^4 |\epsilon(\omega)\epsilon(3\omega)|^2} \left| \chi_R^{(3)} : \epsilon_3 \epsilon_2 \epsilon_2 \right|^2 I(\omega)^3 \left( \frac{r}{\lambda} \right)^6 \int_0^l e^{-(r+S)/\lambda} dS \right)^2, \]

where \( \epsilon(\omega) \) is the material dielectric constant, \( \epsilon_\Omega = L_\Omega \epsilon_\Omega \) indicates the unit polarization vector of the field at frequency \( \Omega \) and \( L_\Omega \) is the Fresnel factor for the field, \( I(\omega) \) is the laser intensity, \( r \) is the radius of the probe tip, \( k \) is a material-specific barrier to tunneling, and \( \lambda \) is the wavelength of the detected photons. The optical transmission efficiency of a subwavelength aperture varies as \( (r/\lambda)^6 \) and signifies a sacrifice of optical signal for high spatial resolution.6,7 The exponential attenuation term models the optical transmission mechanism as a photon tunneling event from the position of the nonlinear field creation event at a distance \( S \) from the near-field probe tip to a region within the probe that is of wavelength dimensions at a distance \( T \) from the probe tip.6,8,9 It should be noted that the source distance (\( S \)) in the exponential term will attenuate signals produced within the detection volume, which is typically approximated as a cylinder of radius \( r \) beneath the tip, as distance from the tip is increased, resulting in the observed depth of field a distance \( l \) into the sample. Not explicitly accounted for in Equations 3 and 4 is the effect of phase matching relaxation that should increase observed signal levels by collecting the nonlinear evanescent electric field prior to cancellation in non-phase matched directions in the far-field.11,12 Additionally, the Fresnel factor for the exiting of the nonlinear signal terms (\( e_{2\omega} \) and \( e_{3\omega} \)) have not yet been systematically studied as to their actual behavior in the near-field.13

The fiber optic tip is mounted in a scanning, non-contact AFM–like system for precise collection of optical signals as a function of position. Maintenance of near-field feedback conditions simultaneously images the physical topography of the sample.

### 4. Experimental

We currently employ a Thermomicroscopes Lumina system that incorporates a stage scanner with 50 \( \mu \)m \times 50 \( \mu \)m horizontal (x-y) and 10 \( \mu \)m vertical (z) scan range. It also features a non-optical, shear-force feedback mechanism that can maintain a constant sample-tip separation near 5 nm.14,15 As shown in Figure 1, laser pulses were focused from the far-field to a ~ (100 \( \mu \)m)^2 spot onto the region of a sample that was investigated by the near-field probe.
Signals collected in the near-field were directed by the fiber optic to filters and a detector which was a 0.3 m spectrograph and CCD for spectra or a photomultiplier tube (PMT) for imaging. Topographical and optical signals were obtained simultaneously in each experiment for comparison. Forward and backwards scans along the same area were collected to produce separate images as a measure of reproducibility. Repeatable optical images could then be added together to reduce random noise. All images in this work are 200 x 200 pixel arrays, required typically 30-35 minutes to collect, and have not been processed in any way.

The light source for all of our NCIN experiments consists of a home-built titanium:sapphire (Ti:S) oscillator (800 nm, 480 mW, 30 fs, 88 MHz) which is used to seed a commercial (Spectra-Physics) chirped pulse amplifier (800 nm, 2.25 W, 80 fs, 1 kHz). This output is beamsplit 90:10 using the 90% beam to pump a commercial (Quantronix, Topaz) superfluorescence optical parametric amplifier (OPA) (1.16-2.7 μm, 300 μJ @ 1350 nm, 80 fs). Tunable mid-IR wavelengths can be produced via difference frequency generation between the signal and idler OPA wavelengths in a AgGaS crystal (2.8-10 μm, 20 μJ @ 4.4 μm, 155 fs). The residual 10% of the amplifier output can also be attenuated and used in SFG experiments. Previously, as in the below SHG and THG measurements, we utilized adiabatically pulled fiber optic probes, however, currently we employ chemically etched probes due to the observed ~ 10x higher signal levels as well as the ability to reduce incident laser power levels at the sample.

5. Second Harmonic Generation NSOM

Far-field SHG imaging has been applied successfully to a variety of chemical problems in far-field studies ranging from materials\textsuperscript{16-18} to biological samples.\textsuperscript{19-21} Of these, biological samples present the most difficulty due to weaker nonlinear responses as well as higher inherent fragility and disorder. Near-field detected SHG, likewise, has been applied to a several samples, ranging from metal films\textsuperscript{22} and piezoceramics\textsuperscript{23} to Langmuir-Blodgett films\textsuperscript{24} and subcellular organelle features.\textsuperscript{25}

We explored the use of nonlinear optics as a noninvasive tool to study organelle features in natural killer cells,\textsuperscript{25} demonstrating chemically selective imaging via selective resonant enhancement of SHG for the first time in NCIN imaging. In these experiments, the results of which are shown in Figure 2, we repeatedly imaged the same area of a natural killer (NK) cell and stepped the wavelength of our amplified, tunable femtosecond laser such that SHG frequencies became resonant with the absorption spectrum of an organelle-specific stain, azure B. SHG images produced with the incident laser tuned to an azure B electronic transition resulted in resonant enhancement of the SHG from only those spatial regions of the NK cell containing cytolytic granules. Furthermore, the highest SHG contrast, i.e. the largest resonant enhancement, shown in part c, was observed at bluer wavelengths than the linear absorption $\lambda_{\text{max}}$ of azure B produced with water as the solvent. Because the dye exhibits a solvatochromic shift to the blue in lower polarity environments as we have shown in part a, we believe that interfacial selectivity was observed in these measurements, the low polarity environment being provided by the granular membrane. Linear reflectivity images produced at the same wavelengths as the detected SHG do not bear semblance to the SHG images which indicates that the SHG contrast does not result from Fresnel factors.
Third-order nonlinear optical probes, such as THG, which can be produced efficiently from both isotropic and anisotropic sample environments, in distinction from second-order measurements, can provide a degree of environmental selectivity in high spatial resolution imaging. Non-resonant THG is developing rapidly as a far-field imaging technique. Much of the interest has been due to the results of Tsang\textsuperscript{26} who first reported what is now often referred to as “surface”
THG, viz. very tightly focused, high intensity (100s of GW/cm²) laser pulses can produce THG signals that are several orders (~10⁵) of magnitude stronger when the focal volume of the laser contains an interfacial region such as air/glass. This result, which is emerging to be a generally observable effect in the far-field non-resonant case, appears to be in stark contrast to the generally accepted view of THG as a bulk probe. The mechanism of this apparent “surface” THG has been proposed to either embody an enhanced surface component of a material’s $\chi^{(3)}$ response, which would imply monolayer-order sensitivity, or may be described by the classically calculated THG efficiency of focused Gaussian beams in a region where either a change in refractive index, $n$, or $\chi^{(3)}$ exists as first purported by Boyd. The latter of these possible mechanisms, which would involve thousands of monolayers and, therefore, constitute a bulk measurement, implies that a change in either $n$ or $\chi^{(3)}$ would assist in relaxing the phase matching condition for the process, resulting in a very significant increase in THG efficiency. In any case, far-field, non-resonant THG has been successfully applied by several researchers to image a variety of samples.

In order to explore the utility of THG as a contrast mechanism in near-field imaging, we performed NSOM-detected THG experiments on samples of human erythrocytes. A-b, THG NSOM images of two coagulated human erythrocytes in a (17 µm)² area produced at $3\omega = 420$ and 490 nm, respectively for the same area shown topographically in c. The maximum topographic height of c is 710 nm, optical pixel $z$-values represent the average of 40 laser shots, incident laser pulse energy was ~ 15 µJ, dark regions correspond to low signals (~ 0 to 1 photon/laser shot) and bright regions correspond to high signals (~ 3 photons/laser shot). As indicated by the linear absorption spectrum shown in d, [Cordone, 1986 #135] THG contrast is very high in a when $3\omega$ is resonant with the Soret transition of oxyhemoglobin (oxyHG) and no contrast is observed in b when the THG is non-resonant. OxyHG, a non-fluorescent, intrinsic chromophore, is a protein that is free in the cytosol, indicating that bulk environmental selectivity is observed, and, therefore, resolution-limited features do not appear in part a. The lack of optical contrast in b (the non-resonant THG measurement) provides evidence that far-field non-resonant THG measurements that are debated to exhibit surface selectivity actually constitute a bulk probe. D, The power dependence of the collected signal through the NSOM fiber probe was measured at $3\omega = 420$ nm. A fit to the data points, each point representing the average of 21,000 laser shots (RMS noise values shown), shows a third order power dependence with fundamental intensity. The data represented logarithmically in the inset shows a slope equal to three linear fit to the data, confirming that detected signals result from the THG process.
erythrocytes and tuned our excitation laser on and off resonance with the absorption spectrum of oxyhemoglobin ($\lambda_{\text{max}} = 415$ nm), a nonfluorescent, bulk chromophore that is free in the cytosol. These experiments probe the utility of THG as a chemically specific optical contrast mechanism, and in the non-resonant case, serve as a test of the two aforementioned proposed sources of far-field, non-resonant THG “surface” enhancement due to the a priori relaxation of phase matching conditions inherent in NCIN. Moreover, they demonstrate the use of intrinsic chromophores for chemically selective imaging.

THG NSOM images of erythrocytes produced at $3\omega = 420$ and $490$ nm are shown in Figure 6a-b, respectively, for the same topographic region shown in c. As can be clearly discerned in these images, high contrast is observable in a in which the third harmonic is resonant with the strong Soret absorption band of oxyhemoglobin as shown in d. While THG signal levels are non-zero in b in which the third harmonic is not resonant with the electronic absorption band of the chromophore, optical contrast between the fused silica substrate and the erythrocytes is not observed. Figure 6e displays the cubic dependence of signal levels collected at the THG frequency on incident laser power, which confirms that the signals result from the THG process. Based upon these results, it appears that THG can indeed produce chemically specific image contrast with bulk environmental selectivity using resonant enhancement from intrinsic chromophores. The lack of optical contrast in the non-resonant THG NSOM image shown between the erythrocytes and the substrate indicates that far-field “surface” selective THG signals result from a relaxation of phase matching conditions at interfaces, and not from intrinsic surface components of the $\chi^{(3)}$ response. This, as explained above, dictates that far-field THG imaging constitutes a bulk spectroscopic probe.

7. Conclusions

We have demonstrated the utility of coherent nonlinear optical spectroscopy for biological imaging with SHG and THG. Resonant enhancement has been demonstrated as a novel contrast mechanism in NSOM imaging that provides chemical selectivity based upon molecular transitions, while environmental selectivity is achieved via the order of the nonlinear process. The THG NSOM measurements also indicate that far-field “surface” selective THG measurements constitute a bulk probe.

8. Acknowledgement

This work was supported by the Experimental Physical Chemistry Division of the National Science Foundation.