Electronic Preresonance Stimulated Raman Scattering Microscopy

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ABSTRACT: Optical microscopy has generated great impact for modern research. While fluorescence microscopy provides the ultimate sensitivity, it generally lacks chemical information. Complementarily, vibrational imaging methods provide rich chemical-bond-specific contrasts. Nonetheless, they usually suffer from unsatisfying sensitivity or compromised biocompatibility. Recently, electronic preresonance stimulated Raman scattering (EPR-SRS) microscopy was reported, achieving simultaneous high detection sensitivity and superb vibrational specificity of chromosomes. With newly synthesized Raman-active dyes, this method readily breaks the optical color barrier of fluorescence microscopy and is well-suited for supermultiplex imaging in biological samples. In this Perspective, we first review previous utilizations of electronic resonance in various Raman spectroscopy and microscopy. We then discuss the physical origin and uniqueness of the electronic preresonance region, followed by quantitative analysis of the enhancement factors involved in EPR-SRS microscopy. On this basis, we provide an outlook for future development as well as the broad applications in biophotonics.

Modern optical spectroscopy and microscopy methods have allowed researchers to study molecular processes in biological systems with unprecedented sensitivity and specificity. In particular, fluorescence microscopy is almost the method of choice for bioimaging applications. It offers robust single-molecule detectability, target specificity, and biocompatibility by probing the electronic resonance of versatile fluorescent probes and detecting the Stokes-shifted emission in a background-free manner.1–4 Complementary to fluorescence, Raman microscopy has become an increasingly valuable bioanalytical tool by providing rich chemical information derived from chemical-bond-specific vibrational transitions (Figure 1). However, the conventional spontaneous Raman scattering process is known to be about $10^{-14}$ orders of magnitude weaker than fluorescence (Figure 2a) and thus is highly restricted in its applications for live-cell imaging.5 In fact, the concern for sensitivity has almost always been associated with linear Raman detection when compared to fluorescence, with only one notable exception. That is the near-field-based techniques of surface-enhanced raman scattering (SERS) and tip-enhanced Raman scattering (TERS), which achieved single-molecule sensitivity about more than 20 and 10 years ago, respectively.6–10 It has long been thought (and debated) that the remarkable enhancement factor (EF) of $10^{10}$–$10^{14}$ for SERS and TERS solely originated from plasmonic enhancement with the metallic nanostructures acting as optical antennas. It was only quantified much later that the resonance Raman effect, involving electronic resonance, played a significant role.11–13 Considering that fluorescence is also a resonant process by definition, it seems that, to ensure high detection sensitivity (possibly down to single molecules), a shared feature here is by going electronic-resonant.

Indeed, the observation of the resonance Raman effect could be traced back to 1950s, even before the invention of lasers.14–16 Later, electronic resonance-enhanced Raman scattering proved to be particularly useful for spectroscopic studies. It could sensitively and selectively probe the chemical structures as well as the excited-state photodynamics by bringing the laser excitation energy ($\omega_{\text{exc}}$) close to the molecular absorption peak ($\omega_{\text{abs}}$) in both deep-UV and visible ranges (Figure 2b).17–19 This strategy exploits electronic and vibrational coupling in chromophores, in which the electronic resonance significantly promotes those nuclear vibrations that are coupled to the electronic transition. Although such a gain of Raman intensity offers good sensitivity for spectroscopic interrogation of light-absorbing chromophores, it often comes with several issues for imaging applications. First, the achievable Raman signals can be easily overwhelmed by the...
required metallic surface in close proximity also effectively deprives the possible fluorescence background. However, as powerful and as sensitive as it is, there are a few near-field associated limitations. Biologically, the strict reliance on metallic nanostructures restricts the general applicability of SERS for biological targets. The close (angstrom-level) contact with the metal surface could often perturb the native properties of biomolecules, such as denaturing the proteins. Physically, SERS is usually difficult for precise quantitative analysis because the EFs offered by the surface plasmons vary substantially between different substrate–molecule configurations. With deeper understanding of the SERS mechanism, it was also gradually revealed that the resonance Raman effect played a significant role in the overall large EF. SERS of many chromophores could hence be more precisely regarded as surface-enhanced resonance Raman scattering (SERRS). Nonetheless, for a long time, the exact contribution of electronic enhancement was less clear due to a lack of proper tools to characterize the electronic resonance factors.

Evidenced by the evolutionary path of fluorescence microscopy, far-field spectroscopy provides more general biocompatibility compared to near-field methods. Nonlinear Raman scattering, the all-far-field advanced Raman spectroscopy, naturally takes the next lead in enhancing the Raman signals and exploiting electronic resonance in pursuit of higher sensitivity. Nonlinear Raman scattering, the all-far-field advanced Raman spectroscopy, naturally takes the next lead in enhancing the Raman signals and exploiting electronic resonance in pursuit of higher sensitivity. Evidenced by the evolutionary path of fluorescence microscopy, far-field spectroscopy provides more general biocompatibility compared to near-field methods.
rations. Later in 2008, triple-resonance coherent antistokes Raman scattering (CARS) microspectroscopy using femtosecond laser pulse shaping was also reported for detecting electronic resonance-enhanced nonlinear Raman signals from nonfluorescent molecules. This method offered a sensitivity approaching 100 molecules in solution with 3 s integration.25

A consensus has been reached in the field that SRS has superseded CARS microscopy in almost all technical aspects. However, the notion of resonance Raman has largely escaped from the radar of the modern nonlinear Raman imaging community, such as for CARS and the more recently developed stimulated Raman scattering (SRS) microscopy (Figure 2c). Both of them have been proven to be highly desirable for label-free imaging with even up to video-rate speed.26–32 A consensus has been reached in the field that SRS has superseded CARS microscopy in almost all technical aspects. In particular, SRS imaging offers linear dependence on concentration and shows identical spectra to spontaneous Raman with no interference complications from the nonresonance background as in CARS.27,28 By virtue of these advantages, SRS imaging, mostly in the label-free form, has made a major impact with exciting applications in biological and medical photonics.28,33–43 Such a label-free strategy is appealing and powerful as it introduces zero physical and chemical perturbation to biological systems. Going beyond the label-free concept, the coupling of SRS microscopy with small and bio-orthogonal vibrational tags, such as alkynes (i.e., carbon–carbon double bond), has been recently demonstrated for the detection of small biomolecules in the cell-silent Raman spectral window.44,45 Such bio-orthogonal chemical imaging offers a powerful platform for functional metabolic imaging in live cells and animals.36–35 Its success underscores the importance of introducing vibrational probes to improve specificity and sensitivity of nonlinear Raman microscopy. However, even with extensive efforts of instrumentation improvement and small-tag optimization, the detection sensitivity of SRS is still in the range of 35 μM (i.e., diyne tags, double-conjugated alkynes)51 to 200 μM (small alkyne tags).34 Here, we note that nearly all of the previous CARS and SRS imagings were operated in the nonresonance region, in which the energy of the pump laser (ωpump) is well below that of the molecular absorption peak (i.e., ωpump ≪ ω0) (Figure 2c).

Realizing the possible large electronic resonance EF of 10^6 when compared to nonresonance Raman signals (e.g., C–O bond), Wei et al. introduced a new scheme of SRS microscopy by shifting from the commonly exploited nonresonance region (Figure 2c) to the electronic resonance (i.e., ωpump close to ω0) (Figure 2d,e).56 First, they directly explored the rigorous resonance SRS detection (Figure 2d). Because the Stokes laser wavelength (λStokes) is fixed at 1064 nm in the setup, the pump laser wavelength (λpump) for detecting typical electronically coupled vibrational modes (e.g., the total-symmetric vibration of conjugated double bonds, at ~1600–1660 cm⁻¹) is around 906 nm. Hence, a far-red absorbing molecule IR895 (λabs ≈ 900 nm) was chosen as a model compound for exploring the rigorous resonance SRS microspectroscopy. An intense but broad peak was observed without clearly identifiable vibrational signatures.56 The calculated cross section for this broad peak is about 10^6 of the standard Raman cross section of C–O bond in methanol acquired under the same SRS laser excitation conditions. Because the SRS signal is detected as pump laser intensity loss (i.e., the stimulated Raman loss) in the presence of Stokes photons, such a huge background does not result from fluorescence emission but a combination of the competing nonlinear optical processes.54,55 It might include other electronic resonance-enhanced four-wave mixing path-

Figure 3. EPR-SRS imaging of specific cellular targets. (a) On-resonance EPR-SRS imaging of ATTO740-labeled 5-ethynyl-2′-deoxyuridine (EdU) by click-chemistry for newly synthesized DNA in HeLa cells, targeting the double-bond vibrational peak of ATTO740 at 1640 cm⁻¹. (b) Off-resonance (1616 cm⁻¹) imaging of the same sample as that in (a) by tuning the pump wavelength away for 2 nm. (c–f) EPR-SRS imaging of ATTO740 immunolabeled α-tubulin (c), Tom20 (d, Mitochondria marker), Giantin (e, Golgi marker), and fibrillarin (f, Nucleoli marker) in HeLa cells. (g) EPR-SRS imaging of methylene blue, a known drug with low fluorescent quantum yield, in live HeLa cells. (h) EPR-SRS imaging of nonfluorescent oxidation product 4,4′-dichloro-5,5′-dibromoindigo from X-gal hydrolysis in live E. coli. Scale bar, 10 μm.
ways such as resonant Rayleigh scattering and the absorption-based pump photon loss due to ground-state population restoration by stimulated emission from Stokes photons.

Comparing the measured EF of $10^8$ from the broad peak of IR895 with the reported rigorous resonance Raman EF of $10^9$, a signal-to-background (S/B) ratio of about 1% might be expected between its narrow-band vibrational signal and the broad-band electronic background. Such a small S/B ratio could be easily buried by background fluctuation. This was indeed the case where the narrow Raman features of IR895 were almost unobservable in the rigorous resonance SRS spectrum. As a reference, the solvent Raman peak of CH$_3$ from pure methanol was still identifiable with a S/B of about 8%. It is worth mentioning that, in the case that Raman features are resolvable under rigorous electronic resonance SRS, normal Lorentzian-shaped Raman peaks should be inversely or partially dispersed depending on where exactly the laser is exciting on the absorption peak of the molecules.

Similar broad electronic backgrounds also exist in FRS spectra beneath the vibrational contrasts. Such a background is not a big issue for spectroscopic characterization with good signal-to-noise ratio but would largely complicate the interpretation for demanding imaging applications, in which unambiguous differentiation of on-resonance and off-resonance (ideally with vanishing contrasts) contributions is essential. Because the off-resonance background carries noise and is usually not spectrally flat beneath the on-resonance signal, a simple on-off subtraction may not work well for imaging analysis.

If rigorous resonance as in IR895 quickly brings up the background by evoking a combination of electronically enhanced multipathway backgrounds, would proper detuning away from the rigorous resonance help attenuate the electronic background and hence restore the chemical selectivity? Wei et al. reported an electronic preresonance SRS (EPR-SRS) scheme by shifting the excitation to an electronic preresonance (EPR) window, in between the rigorous resonance and the nonresonance regions. In this scenario, the pump laser is exciting on the absorption peak of the molecule. It was shown that 97% of the fluorescent quantum yield chromophores could be probed by EPR-SRS with good sensitivity and high selectivity. Because the off-resonance background carries noise and is usually not spectrally flat beneath the on-resonance signal, a simple on-off subtraction may not work well for imaging analysis.

$$\sigma = K\omega_p(\omega - \omega_0)^2\left(\frac{\omega_0^2 + \omega_p^2}{\omega_0^2 - \omega_p^2}\right)^2$$

where $\omega_p$ is the electronic transition energy and $K$ is a collection of frequency-dependent factors of the Raman dyes. This equation could well describe the EPR-SRS measurements on dyes across a wide spectrum of absorptions by assuming dye-independent oscillator strength and ground-to-excited state Franck-Condon factors. We note that here the EPR-Raman cross sections are dependent on the frequency detuning ($\omega_0 - \omega_p$) to its fourth power.

As a comparison, the molecular absorption spectrum in the condensed phase may be modeled by a pseudo-Voigt profile

$$V(\nu) = (1-f)L(\nu) + fG(\nu)$$

where $L(\nu)$ is the Lorentzian distribution for homogeneous line broadening and $G(\nu)$ is the Gaussian distribution for inhomogeneous line broadening. Taking the measured absorption spectrum of ATTO740 in solution as a reference, $\Gamma_e = 670$ cm$^-1$ and $f = 0.98$ best fit its absorption profile, largely following a Gaussian distribution, consistent with the known conclusion that absorption spectra in solution are largely inhomogeneously broadened. We then overplotted eqs 1 and 2 and normalized the values in reference to the numbers detuned away by 1.5$\Gamma_e$. It became obvious that the Raman signal from eq 1 decays much slower than the absorption signal (eq 2) in the preresonance region of the experiment (Figure 4, 820 nm (detuned away by 2$\Gamma_e$) to 920 nm (detuned away by 4$\Gamma_e$)). This comparison illustrates that virtual-state mediated preresonance Raman processes follow a slower decay as a function of frequency detuning in the defined preresonance regime compared to real-state mediated absorption transitions.

Qualitatively, such slower signal decay behavior of EPR-SRS might also be understood by the frequency detuning dependence of absorption and Raman on $\chi^{(1)}$ and $\chi^{(3)}$, respectively. For the EPR-SRS process, under a strong
frequency detuning (eq 1), a qualitatively similar dependence of resonant Raman (the imaginary part of eq 4) versus linear dispersion (the real part of eq 5) on frequency detuning could be clearly spotted. Therefore, the differential frequency dependence between resonant Raman and linear absorption is analogous to the more familiar relationship between dispersion and absorption, in which the real part of $\chi^{(1)}$ (dispersion) is well-known to decay much slower compared to the imaginary part of $\chi^{(1)}$ (absorption) after a certain detuning range (e.g., in the EPR range) (Figure 5). Although the supermultiplex capability of the EPR-SRS method could go beyond imaging to generating broad impacts in other fields of photonics, such as flow cytometry and data security.

We next quantitatively analyze the EPR-SRS cross sections and the essential contributing factors for comprehensively understanding and better developing EPR-SRS microscopy. The spontaneous Raman cross section for the standard C–O vibration at 1030 cm$^{-1}$ in methanol ($\sigma_{\text{spon, Raman}}$ (C–O)) was reported to be $2.1 \times 10^{-50}$ cm$^2$ under 785 nm excitation. Extrapolating from 785 nm using the $\omega_{\text{pump}}\sigma_{\text{spon, Raman}}$ dependence to the SRS excitation wavelength of $\lambda_{\text{pump}}$ $\approx$ 960 nm and $\lambda_{\text{Stokes}}$ $\approx$ 1064 nm, $\sigma_{\text{spon, Raman}}$ (C–O) becomes $0.9 \times 10^{-30}$ cm$^2$. As shown in Figure 6a, the measured SRS signal of C–O in pure methanol (~24.7 M in concentration) is $\approx 1.7 \times 10^{-4}$ under a Stokes laser power ($P_{\text{Stokes}}$) of 120 mW. With a laser excitation volume of about $2 \times 10^{-15}$ L, 24.7 M corresponds to $3 \times 10^9$ C–O bonds in the laser focus. With a laser waist area of about $2 \times 10^{-9}$ cm$^2$, the relative spontaneous Raman signal ($\Delta I_{\text{spon}}/I$) becomes comparable to that estimated before by both SRS microscopy

Figure 4. Signal dependence on the pump laser wavelength (also the frequency detuning ($\Delta \omega_{\text{pump}}$)) Electronic pre-resonance Raman (blue) and absorption (red) spectra, normalized to 1.5 times the absorption maximum.

Figure 5. Dependence of the imaginary part (black line, i.e., absorption) and the real part (blue line, i.e., dispersion) of $\chi^{(1)}$ on the laser frequency ($\omega_{\text{pump}}$) detuning from the electronic transition energy ($\omega_{ge}$).

The supermultiplex capability of the EPR-SRS method could go beyond imaging to generating broad impacts in other fields of photonics, such as flow cytometry and data security.
Figure 7. Representative eight-color tandem EPR-SRS and fluorescence imaging on the same set of neuronal cells. EPR-SRS targets: HPG (a, red, L-homopropargylglycine, for proteins synthesized in the pulse period for 12 h, labeled with MARS2228); AHA (a, green, 1-azidohomoalanine, proteins synthesized in the chase period next for 10 h, labeled with Alexa 647); βIII-tubulin (b, gray, neurons, labeled with MARS2200); myelin basic protein (c, MBP, orange, oligodendrocytes, labeled with MARS2176); and glial fibrillary acidic protein (c, GFAP, magenta, astrocytes and neural stem cells, labeled with MARS2147). Fluorescence: NeuN (b, blue, neurons, labeled with Alexa668); LipidTux (b, cyan, lipid droplets); Nucblue (c, yellow, total DNA). Scale bars, 10 μm.

We believe that EPR-SRS microscopy together with the newly developed MARS palette could become a valuable systems-method helping to elucidate complex biochemical and biophysical processes.

In retrospect, many biological discoveries were driven by technical innovations that explored less-charted spectroscopic principles, with assistance from novel matching reporters. The previously less-explored EPR-SRS imaging exhibits the desired combination of sub-μM high sensitivity and narrow chemical selectivity, merging the best of two worlds of electronic and vibrational microspectroscopy. We believe that EPR-SRS microscopy together with the newly developed MARS palette could become a valuable systems-method helping to elucidate complex biochemical and biophysical processes. Therefore, we hope this Perspective contributes to a deeper and more quantitative understanding of the fundamental physical principles underlying this new technique and help push its further development.

colors. Toward this goal, Wei et al. then devised a novel vibrational palette, in which each Raman-active dye bears a conjugated triple bond (i.e., nitrile or alkylene) and presents a single narrow EPR-SRS peak in the desired cell-silent Raman spectral window. This series of new dyes is termed MARS (MAnhanttan Raman Scattering) dyes. Using the newly developed MARS dyes in the near-infrared (~650–800 nm) and merging with the orthogonal fluorophores in the visible range (~400–650 nm), eight-color imaging on the same set of neuronal cell samples is demonstrated (Figure 7). This number could be further increased with future engineering of dye molecules to provide higher signals as well as better-resolved Raman peaks. Moreover, the supermultiplex capability of the EPR-SRS method could go beyond imaging to generating broad impacts in other fields of photonics, such as flow cytometry and data security.
Methods

We are grateful for discussions with Lixue Shi, Zhixing Chen, Louis Brus, and Sunney Xie. W.M. acknowledges support from NIH Director’s New Innovator Award (1DP2EB016573) and R01 (EB20892) and the Camille and Henry Dreyfus Foundation.

Acknowledgments

We gratefully acknowledge discussions with Lixue Shi, Zhixing Chen, Louis Brus, and Sunney Xie. W.M. acknowledges support from NIH Director’s New Innovator Award (1DP2EB016573) and R01 (EB20892) and the Camille and Henry Dreyfus Foundation.

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