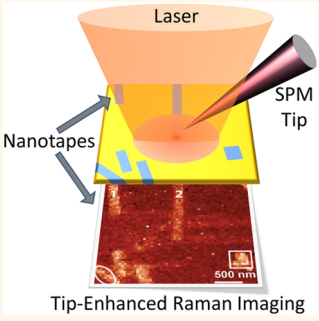
Tip-Enhanced Raman Imaging: An Emergent Tool for Probing Biology at the Nanoscale

Eric A. Pozzi,[†] Matthew D. Sonntag,[†] Nan Jiang,[†] Jordan M. Klingsporn,[†] Mark C. Hersam,[†,‡] and Richard P. Van Duyne[†,§,*]

Departments of [†]Chemistry, [§]Biomedical Engineering, and [‡]Materials Science and Engineering, Northwestern University, Evanston, Illinois 60208, United States

ABSTRACT Typically limited by the diffraction of light, most optical spectroscopy methods cannot provide the spatial resolution necessary to characterize specimens at the nanoscale. An emerging exception to this rule is tip-enhanced Raman spectroscopy (TERS), which overcomes the diffraction limit through electromagnetic field localization at the end of a sharp metallic tip. As demonstrated by the Zenobi group in this issue of *ACS Nano*, TER imaging is an analytical technique capable of providing high-resolution chemical maps of biological samples. In this Perspective, we highlight recent advances and future applications of TER imaging as a technique for interrogating biology at the nanoscale.



Imaging the topography of surfaces with nanoscale spatial resolution is possible with scanning probe microscopy (SPM), which is extremely versatile and can operate in vacuum, air, or liquid on a wide array of surfaces. Under appropriate conditions, non-contact atomic force microscopy (NC-AFM)[1] and scanning tunneling microscopy (STM)[2] can achieve atomic-scale resolution of single molecules and crystalline surfaces. However, SPM topographic imaging is not well-suited for chemical identification. In contrast, optical spectroscopy is an essential tool for probing the chemical signatures of molecules, albeit when imaging with spatial resolution that is typically set by the optical diffraction limits. To minimize the trade-off between spatial resolution and chemical sensitivity, recent work has attempted to integrate SPM and optical spectroscopy with an eye toward achieving both atomic-scale topographic and single-molecule vibrational information. For example, tip-enhanced Raman spectroscopy (TERS) provides chemical information for adsorbed species without sacrificing the molecular resolution of SPM.[3]

In TERS, an intense and confined electromagnetic field at the tip apex leads to amplification of the Raman signal by more than a factor of 10^6. With this large enhancement, resonantly excited dye molecules can be detected at the single-molecule level with TERS.[4,5] As a scanning probe technique, the high spectroscopic sensitivity of TERS is complemented by excellent spatial resolution. Since the localization of the enhanced electromagnetic field is dependent on the dimensions of the tip apex, the lateral spectroscopic resolution is no longer determined by the diffraction-limited laser focus but by the size and shape of the tip. In this manner, spatial resolution in TERS has been reported down to 15 nm.[4,6]

Together, the combination of high sensitivity and nanoscale spatial resolution has made TERS a viable technique for studying weakly scattering samples including those with biological significance. Several relevant systems have been studied including nucleic acids, proteins, bacterial cells, viruses, and human cells, as summarized in previous reviews.[7−9] Although these early TERS-based studies of biological systems have predominantly been performed on noble metal surfaces, different environments have recently been demonstrated such as oxides,[10] glass,[11] and aqueous conditions.[11]

While all of these reports have broadened the applicability of TERS to a wide variety of substrates and systems, one of the more underutilized capabilities of TERS is spectral mapping of surfaces. Initial studies on TER

* Address correspondence to vanduyne@northwestern.edu.

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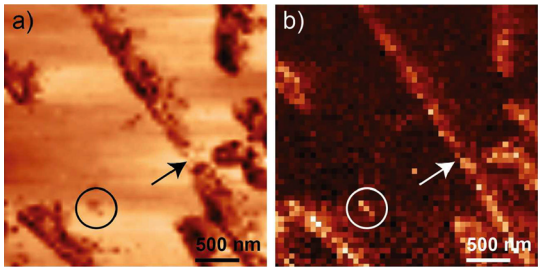

Figure 1. Simultaneously acquired (a) STM and (b) TER (acquisition time 1 s/pixel, 2 mW incident power) images of individual nanotapes with 3 × 3 μm^2 scan size and 50 × 50 pixels. The color-coded TER images display the intensity (higher intensities are represented by brighter pixels) of the aromatic ring breathing marker band (1004 cm^{-1}) value of the peak integral. The arrow and circle illustrate that areas weakly observed as features in the STM image can be identified as nanotape/protein structures using TER imaging. Adapted from ref 13. Copyright 2013 American Chemical Society.

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mapping focused on comparing topographic images to point spectroscopic studies obtained at different locations on the sample.[12] Current techniques have advanced to full spectroscopic imaging of surfaces in which SPM topographic and TER spectroscopic information are simultaneously collected.

The article from the Zenobi group in this issue of *ACS Nano* reports complementary topographic and TER images of peptide nanotapes.[13] The TER images were compiled using the intensity of a known peptide Raman band at each pixel location, thereby enabling chemical identification of features observed in the corresponding topographic images (Figure 1). In addition, features not apparent *via* STM were visualized in TER images. Specifically, a nanotape not evident in the STM image is apparent in the TER image.

Tip-enhanced Raman images of other biological systems in the literature include supported lipid domains,[14] biotin–streptavidin complexes,[15] osteopontin on calcium oxalate crystals as a kidney stone inhibition model,[16] and human colon cancer cells.[17] In the study on human colon cancer cells by Deckert and co-workers,[17] Raman images were not constructed by the absolute intensity of a single peak but instead by the correspondence of each pixel's spectrum to extreme spectra. These so-called endmembers were TER spectra exhibiting strong protein character or strong lipid character. Such images utilize the entire Raman spectrum to distinguish between species, but the number of species present must be known or additional algorithms are required. Choosing a method for TER image construction then depends on the number of species under study as well as their spectral similarity.

These studies set the stage for multianalyte TER imaging exploiting the unique vibrational fingerprints of each analyte. If distinct membrane proteins of similar size and shape exist in a lipid bilayer, for example, conventional SPM cannot discriminate among the proteins. On the other hand, mapping peak intensities of a Raman band distinct to one protein will produce an image in which only that species is visible. Analogous images can be constructed for each protein type present. Multiple bands for each species can then be used to produce TER images, which, upon agreement, will strengthen the characterization. All of this information can be produced by a single TERS scan of the membrane since the entire Raman spectrum is collected at each pixel. Consequently, this technique can, in principle, enable the full characterization of a realistic membrane containing numerous protein constituents.

Given its high spatial resolution and sensitivity, TERS has also been suggested as a label-free DNA sequencing tool. Reports of TERS on crystals or monolayers of individual nucleobases date back to 2004.[18,19] More recently, TER spectra have been reported for a single RNA homopolymer of cytosine[20] and for a calf thymus DNA strand.[21] Spectra of natural, heteropolymeric DNA show many Raman bands, as expected, but specific marker bands for each nucleobase can be identified.[21]

Bailo and Deckert have proposed a sequencing procedure for straightened single strands using TERS (Figure 2).[20] If the spectrum at a point along the strand contains contributions from multiple adjacent bases, the tip can be moved along the strand by a distance equal to one base-to-base length, at which point another spectrum can be acquired. Spectral differences can then be attributed to nucleobases entering and exiting the tip's enhancing region. Alternatively, DNA or RNA strands may be sequenced using TER imaging. If a TER image of a single strand is obtained with high enough pixel density, spectra from individual pixels corresponding to tip positions over the strand as determined by SPM can be identified and analyzed in an analogous manner.

OUTLOOK AND FUTURE CHALLENGES

Interpreting TER spectra and assigning vibrational modes is a major obstacle for biologically relevant

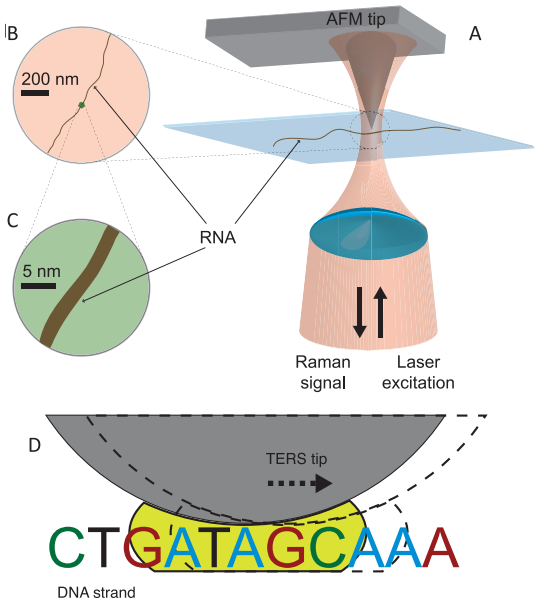

Figure 2. Direct base-sequencing (A) schematic and (D) procedure using TERS. (B) Magnified region roughly equal to the focal area of the laser beam spot. (C) Further magnified region approximating the enhancing region of the tip. (D) Sequence information can be obtained by laterally shifting the probe in intervals of one base-to-base distance. The yellow area refers to the enhancing region at the first tip position. Adapted with permission from ref 20. Copyright 2008 Wiley.

TERS studies. Except for special cases, such as selective detection of cyctochrome *c* in mitochondria due to resonance enhancement,[22] TER spectra of biological systems are often complicated because of the number of distinct chemical species present.

Many factors in a TERS experiment can affect the vibrational spectra. The intense electromagnetic near field at the tip apex can perturb the system and cause different selection rules to apply.[23] It has also been shown that the physical force exerted by the TERS probe can cause changes in the vibrational spectra.[18] Because a very small quantity of analyte is detected in TERS, shifts in the TER spectra can occur due to differences in the local environment. Further complicating data analysis, TERS peak positions can be shifted by changes in the distance between the probe and the molecule.[20,24] In addition, spectral contributions from the sample surface and any contamination from sample preparation must be accounted for in TERS studies.

Tip-enhanced Raman spectroscopy sequencing of DNA/RNA requires careful spectral analysis because multiple nucleobases, with varying Raman cross sections, are present in the enhancing region at once. This process can be further complicated because it has been shown that shifts in the expected vibrational spectra can occur from interactions within the DNA or RNA molecule.[25] Therefore, spectral analysis of such complicated systems requires sophisticated data analysis techniques. For example, principal component analysis is one useful tool for identification when multiple analytes are present, but it must account for small changes in the TER spectra over time. As TER imaging apparatuses and data analysis techniques improve, increasingly realistic and complex biological systems will be studied, which will increase the prevalence of TER imaging as a practical and powerful tool for researchers in the biosciences.

Conflict of Interest: The authors declare no competing financial interest.

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