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FIRST REACTIONS

# Azo Pigments Make Raman Spectral Multiplexing More Sensitive

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**An azo-conjugated strategy to enhance Raman signals of vibrational bonds proves the giant potential of molecular engineering for ultrasensitive Raman multiplexed detection.**

Azo pigments have a history of nearly 150 years of usage in the dye industry. Azo derivatives like azobenzene are now considered molecular switches because of photochromism, thereby attracting a broad interest in many fields of modern chemistry, materials science, and biology.[1] In this issue of *ACS Central Science*, Tang et al. revealed a unique resonance Raman enhancing effect of azo pigments on molecular vibrations.[2] A library of azo-coupling vibrational probes with tunable Raman frequencies and ultrahigh sensitivity has been engineered to fulfill spontaneous Raman multiplexed bioassays in complex systems.

Biological systems are complex. A basic goal to understand biological processes is deciphering molecular information and events in cells, which often requires a high-throughput multiplexed solution. One of the most popular methods currently is using fluorescent tags to label biospecimens and decoding the information through microscopy. Even though this method has successfully been used in exploring structure–function relationships in cells and tissues,[3] it is challenging to analyze many species with high selectivity and sensitivity under biological conditions. Typical tags such as organic dyes often emit broad spectra, which limit the number of resolvable colors from two to five—a situation known as the "color barrier".[4] Materials such as quantum dots and lanthanide nanocrystals were developed to resolve this barrier by leveraging their intrinsic narrower line widths or creating external information code dimensions,[5] but new complications have emerged. For instance, their relatively bulky size may limit the accessibility of particles to the biomarkers of interest and even alter the biological activities of an object.

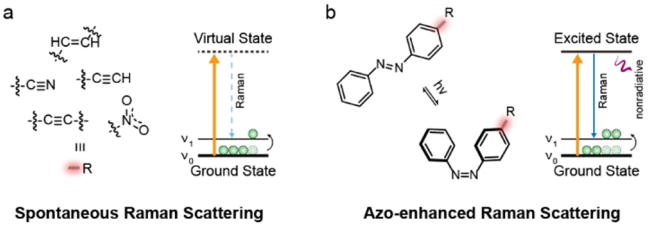


**Figure 1.** Principle of azo-enhanced Raman scattering (AERS). (a) Spontaneous Raman scattering of a specific chemical bond is weak because of the low cross section of the virtual electronic energy level. (b) In AERS, highly efficient vibrational excitation can occur as a result of the resonance Raman effect by conjugating an azo unit. Meanwhile, the nonradiative decay of azobenzene quenches the commitment fluorescence, thus providing a clean background for identifying Raman scattering.

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Raman-based vibrational microscopy represents an alternative approach for elucidating bioassays. In principle, unlike fluorescence, Raman scattering detects the vibrational signatures of molecules. The signatures depend on the specific type of chemical bonds, and each of them has an intrinsic resonant frequency (Figure 1a), which is the foundation of Raman microscopy as a label-free imaging method.[6] The vibrational signals have a unique merit of much narrower line widths (peak width ~10 $cm^{-1}$) compared to fluorescence,

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which could dramatically increase detection throughput. The only fly in the ointment is that spontaneous Raman signals are too weak (about $10^{-3}$ to $10^{-4}$ of the intensity of the exciting source). This substantially limits the detection sensitivity and imaging speed, as acquiring sufficiently strong Raman signals for imaging in biological specimens requires millimolar concentrations of molecules and longer pixel dwell times than fluorescence spectroscopy-based methods. In an effort to address this challenge, many new techniques and strategies have been developed. One of the most important achievements in recent years is electronic preresonance stimulated Raman scattering (EPR-SRS) microscopy.[7] This technique relies on a customized library of exogenous synthetic probes called MARS dyes, which were created by conjugate coupling of rhodamine fluorophores with chemical bonds with specific vibrational modes (Figure 1b). Subsequently, electronic resonance excitation greatly enhances Raman signals, but careful adjustment through detuning is necessary to prevent fluorescence from overwhelming the enhanced Raman signals. In addition, the facility of EPR-SRS microscopy requires high-cost lasers and sophisticated optics, which are not readily accessible to broader researchers to work with.

If the vibrational bond conjugated fluorophore is nonfluorescent, for example, a dark quencher, one may also expect to obtain a great contrast between resonance Raman scattering and the electronic background. Tang and co-workers developed a concept called azo-enhanced Raman scattering (AERS). Inspired by the dark quenching characteristics of the azo moiety, they have developed a new kind of vibrational probe by conjugate coupling of azo pigments with various vibrational bonds. They initially conjugated azobenzene to the polyynes and systematically investigated the structure−property relationship to reveal the enhancing factors of $\nu$ (C≡C) Raman bands in the silent frequency region. It has been shown that azo directly conjugated to polyyne enhanced the stretching vibrational signals of $\nu$ (C≡C) by a factor of about $10^2$, due to the resonant Raman effect. Extending the $\pi$-conjugated system and improving molecular symmetry by conjugating two or more azo groups into molecules also resulted in Raman enhancement, albeit less than the resonance effect. The most intensive molecule showed about $10^4$ times enhancement of Raman scattering as compared to that of EdU's (5-ethynyl-2′-deoxyuridine, a well-known, small-molecule vibrational tag) alkyne peak.[8] This enabled a linear detection range of 1.0−500 $\mu$M—efficient for potential applications of quantifying molecules of interest.

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Tang et al. further showed that AERS was amenable on eight different groups of vibrational modes including (C≡C), $\nu$ (C≡$C_H$), $\nu$ ($C_{Ph}$–N), $\nu$ (C═C), $\nu$ (C≡N), and $\nu$ (O–N═O). Not only that, they also found that azo conjugation shifted the Raman frequency of specific vibrational modes by varying degrees thanks to the $\pi$-conjugation extension of azo coupling. Importantly, these vibrational modes contain Raman signals in the fingerprint frequency regions, in which signal interference typically exists in the biospecimen. This will allow Raman labels to be easily identified in bioassay experiments and will facilitate the simultaneous use of many different vibrational channels. The authors, therefore, demonstrated the power of their AERS probes as multiple-color cell stains and codes based on spontaneous Raman scattering. Using a mixture of AERS labels, six-color cell images and seven-plex cell coding in total have been identified on a commercial confocal Raman microscope (Figure 2), thereby validating the feasibility of the AERS method.

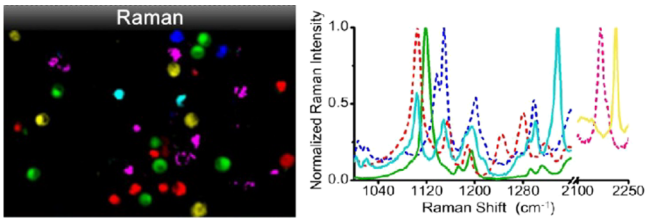


**Figure 2.** Multiplex AERS coded images of mixed suspended HeLa cells (left). Spectral decoding was based on the individual Raman spectrum of each AERS probe (right). Reproduced with permission from ref 2. Copyright 2021 The Authors. Published by American Chemical Society.

The ability to simultaneously identify an increasing number of molecular species in one sample will undoubtedly expand our understanding of biological complexity. Common techniques known as flow cytometry, immunofluorescence, and fluorescence in situ hybridization (FISH) have been key to diagnosing virus infections, tumor histopathology, and other hematological diseases.[9] Ever since the proof-of-principle demonstration of supermultiplex Raman imaging (system with up to 16 vibrational colors),[7,10] there is no doubt that Raman scattering would be an important alternative


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to fluorescence for multiplexed bioassays. A recurring theme in all these exciting developments is the creation of a bright color palette that is resolvable from each other in the optical spectrum. Although it remains to be seen how widely adopted Tang and co-workers' method will be, this work demonstrates the giant potential of molecular design for ultrasensitive Raman multiplexing. These vibrational tools also seem to be compatible with other Raman enhancing techniques such as SRS. It is well worth looking into how many miracles this combination can create and how this combination can accelerate biodiscoveries.

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### Notes

The authors declare no competing financial interest.

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