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Single-Molecule Chemistry is More than Superresolved Fluorescence Microscopy

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In 1982, the scientific community was stunned by the first scanning tunneling images of single atoms on surfaces. But it was also obvious that detecting single molecules in condensed matter would bring the advantages of remote, in-depth, and noninvasive probing. However, in the early 1980s, optics were still limited by Abbe's diffraction limit, which is about a thousand times larger than atomic scales. The solution to this problem was to use optical resonance, that is, to match not only the molecule's position with the laser spot, but also a molecular transition energy with the laser photon energy. If the concentration of resonant molecules is so low that at most one of them may be found in the diffraction-limited spot, a single molecule will interact with the laser light. Such experiments first succeeded in 1989 at cryogenic and 1990 at room temperature, and soon developed into a fully fledged field, namely single-molecule optics.

After 25 years of optical experiments on single molecules, it is time to reflect on the insights and the present or potential applications that single-molecule optics, and more generally single-molecule chemistry, have brought to us. For a broad audience, single-molecule experiments have been merely one of the roads leading to superresolved fluorescence microscopy, or nanoscopy, as highlighted by the Nobel Prize in Chemistry 2014, which was awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner for "the development of superresolved fluorescence microscopy" (see also the Nobel Prize winners' Reviews based on their lectures in Stockholm that are published in this issue).

The saying "seeing is believing" echoes the emphasis that our modern culture puts on pictures and movies. While the importance of images to uncover and understand the structure and mechanisms of complex and especially biological matter can never be overstated, images by themselves don't tell reliable stories: to decipher their meaning—their secrets—both interpretation and careful thought are needed. There is much more to single molecules than the superresolved fluorescence spots that meet our eyes, thanks to laser and image-processing technology. Subtle and detailed information can be gleaned from the wealth of signals single molecules relay to us from their nanometer-scale environment, be it physical—electric and magnetic fields, temperature, pressure, stress—or chemical—pH, redox potential, sensing of small molecules—or symmetry breaks. Let me illustrate this statement with some success stories showing which new information single-molecule experiments provide.

Surprising Insights

The dynamic character of molecular processes is notoriously hard to convey through images, even in the form of movies. Spatial heterogeneity, which is directly visualized in superresolved images, takes its full significance only when the time variable is associated to space. For example, the individual steps of molecular motors can be resolved in time as well as space, or single dye molecules can be tracked as they diffuse in the complex geometries of porous oxides or catalyst surfaces. The occurrence of statistical fluctuations means that the whole field of dynamics needs time traces and correlation functions for quantitative evaluation, particularly when evolution rates are spread over broad scales, with some of them much shorter than video rates. Single-molecule signals present the unique advantage of directly yielding time dependences without any need for ensemble synchronization. In many cases, single-molecule studies have revealed the amazing dispersion of reaction rates in real systems where the traditional paradigm was that of well-stirred reaction solutions and simple low-order kinetics with well-defined, constant reaction rates. This time-resolved information is difficult, if not impossible, to obtain from ensembles. Single molecules give direct access to that heterogeneity at a fixed position as a function of time, or at different positions with spatial correlations.

The dispersion of rates can become extreme wherever high or broad barriers must be crossed for a reaction to occur. A striking example is found in the study of single enzymes pioneered by X. Sunney Xie and H. Peter Lu, known as single-molecule enzymology. Following one enzyme molecule for extended periods of time, they discovered large changes in the turnover rate, giving rise to a succession of "busy" and "lazy" periods. These surprising observations open profound questions about the working of enzymes, and more generally about how molecular machines not only steer clear of, but literally harness the violent perturbations caused by Brow-

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nian motion at nanometer scales. Isn't the rugged free-energy landscape of protein dynamics, first proposed by Hans Frauenfelder, required to preserve the protein's structural integrity? Might the hierarchy of barriers between different conformations be a necessary consequence of a compromise between structural integrity and the flexibility required for proper function? Does a random walk in such a potential optimize the overall turnover rate? Similar considerations may also apply to complex systems in condensed soft matter, such as glasses, whose structure and dynamics remain an old and vexing problem.

A new development in spectroscopy is the application of ultrashort pulse techniques to the dynamics of single molecules at room temperature. Pump–probe experiments with sub-picosecond time resolution by Niek van Hulst's group have demonstrated the unexpectedly long coherence lifetime of vibronic transients measured on single dye molecules. More recently, the same group extended these experiments to bacterial light-harvesting complexes, and again found surprising long-lived quantum coherence even under physiological conditions. Quantum coherence influences excitation amplitudes on different parts of a complex and thereby may strengthen energy transfer pathways against thermal perturbations and disorder, eventually helping photosynthetic efficiency. Such insights might well hold great promise for photovoltaics.

As nearly isolated quantum systems, single molecules have been used to test all kinds of quantum manipulations. Many of the experiments done currently with nitrogen-vacancy (NV) color centers in diamond or with semiconductor quantum dots were first demonstrated with single organic molecules. Several examples of quantum manipulations with single molecules have been demonstrated in the past ten years by Vahid Sandoghdar and his group. At cryogenic temperatures and in high-quality molecular crystals, a guest molecule's optical transition is largely shielded from environmental perturbation during its spontaneous emission lifetime, conferring to it a quality on par with that of a cold atom in gas phase. This feature is unique to molecules among condensed-matter systems. Molecules could thus turn out to be the missing key transducers of quantum information between matter qubits and the flying qubits of photons.

Being very small, single molecules can be used as pointlike probes of optical near fields at nanometer scales, such as those produced around plasmonic nanostructures. One of the most attractive effects in plasmonics is fluorescence enhancement, which lies at the heart of the most resounding commercial success of single molecules so far, namely DNA sequencing in zero-mode waveguides. While DNA is assembled by a DNA-polymerase immobilized in a hole in an aluminum film, the fluorescence of the successively processed dye-labeled nucleotides is enhanced by metal plasmons. The detected signals provide the sequence of base pairs. Another exciting potential application is surface- and tip-enhanced Raman scattering, which provides chemical information down to the single-molecule level. Once reproducible substrates and binding methods have been demonstrated, this approach is poised to become a standard analysis tool. Similarly, fluorescence enhancement by various metal nanostructures has the potential to generalize single-molecule fluorescence to a much broader range of weak or slow emitters. This would considerably shorten fluorescence lifetime, the main bottleneck in single-molecule dynamical studies.

Although fluorescence will remain the workhorse method of single-molecule and single-nanoparticle optical studies for years to come, other optical signals have proved applicable as well. In neatly fabricated flow cells or capillaries, small metallic or dielectric particles can be detected by scattering, even in the presence of background, down even to single protein molecules. Absorbing species can be detected with high sensitivity by photothermal contrast, and plasmonic near fields can be applied for sensing single molecules, either through enhanced absorption and emission, or just through their refractive-index contrast with the surrounding medium.

The Mind's Eye

Far from being merely a route to nanoscopy, single-molecule chemistry has spawned a broad range of original methods, providing subtle information beyond spatial arrangement. These methods have brought an uninterrupted series of insights, among them the surprising extent of heterogeneity at the nanoscale. Where zooming in on single molecules should have been a drastic simplification, nature still baffles us by appearing as intricate as ever at submicrometer scales. It is a safe bet that the single-molecule revolution is not over yet. The whole toolbox of single-molecule methods, used to disentangle chemistry at small length and time scales, is still in need of finer and faster instruments. Experiments on single nano- and subnanoparticles will allow not only seeing, but also mechanistic understanding of more complex systems. Manipulation of nanoparticles by light, as recently demonstrated with optically driven nanoswimmers, will become commonplace. The next step will be the combination of these precise tools with the images from nanoscopy. To slightly paraphrase the fox in Antoine de Saint-Exupery's *The Little Prince*: it is only with the *mind* that one can see rightly; what is essential is invisible, *even* to the *superresolving* eye.

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