Single-Molecule Absorption

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Photothermal Contrast Reaches Single-Molecule Sensitivity

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In 1989 Kador and Moerner reported on the successful measurement of the optical spectrum of a single pentacene molecule doped into a *p*-terphenyl crystal.[1] Notably, in this first account, an absorption experiment was described. Ultrasensitive absorption measurements are intrinsically difficult, because a small number of missing photons has to be discriminated against the photon noise from the light beam. The particular measurement by Kador and Moerner profited from a sophisticated double-modulation technique with quantum-limited sensitivity and the very large peak absorption cross section of the molecule. This last feature was obtained by cooling the sample to 1.4 K under which conditions a very sharp and intense zero-phonon line of the pentacene molecule is generated. Soon after this event Orrit and Bernard employed fluorescence excitation spectroscopy on the same system and achieved a substantial increase in signal-to-noise ratio.[2] Other early attempts to detect single molecules also relied on the efficient detection of the fluorescence emission as a result of its low background.[3,4] After more than 20 years of single-molecule spectroscopy most of the methods developed—including the more recent super-resolution techniques[5]—and most of the results obtained have rested on fluorescence detection.

Nevertheless, since the pioneering work of Moerner a number of different approaches have been pursued to measure single-molecule absorption.[6–8] To date, they also relied on low temperatures and intense zero-phonon lines, and were, in a way, improved versions of the original experiment. To measure single-molecule absorption at room temperature is even more demanding than at low temperature, because the absorption cross section (at a particular excitation wavelength) is reduced by several orders of magnitude compared to the low-temperature value. Hence, the ratio between the absorption cross section at room temperature (ca. 10^{-16} cm^2) and the typical size of a diffraction limited laser spot (ca. 10^{-9} cm^2) is a small number which renders the interaction between the light beam and the molecule weak. Consequently, it becomes very challenging—though not impossible (see below)—to detect the small number of photons absorbed by the molecule ("missing photons") against the noise of the laser beam in a standard transmission-type experiment. Other obstacles are surface roughness and slight variations in the refraction index of the sample. These inhomogeneities scatter light, an effect which may be difficult to distinguish from direct absorption by the molecule under study. It is mainly these points which led Orrit and co-workers to choose photothermal contrast when striving for single-molecule sensitivity.[9]

In photothermal contrast, light from a pump beam is absorbed by the illuminated object. The absorbed energy is converted into heat which is released in the surrounding and creates a thermal lens. The concomitant local change in refractive index $\delta n/\delta T$ is measured by a probe beam with an interferometric set-up. Using an elaborate version of this principle, Orrit and co-workers originally had shown that small gold nanoparticles, down to diameters of 5 nm, could be detected and imaged in a microscope.[10] Such particles, however, are much easier to observe than a single organic dye molecule, because of their much larger absorption cross section. To achieve single-molecule sensitivity, the experimental conditions had to be improved further.[9,11] A number of potential noise sources were eliminated by modulating the pump beam at a frequency of around 1 MHz. In recent work the authors had shown that dissipated powers as low as 3 nW (3×10^{-9} W) could be detected with an signal-to-noise-ratio (SNR) of 8 in a 100 Hz bandwidth.[11] To dissipate the maximum amount of power absorbed by a molecule, absorbers with large non-radiative transition rates and correspondingly low fluorescence quantum yields were investigated, that is, radiative decay by fluorescence is negligible in these molecules. After evaluating a couple of suitable candidates Gaiduk et al. chose a so-called black hole quencher (an azo dye molecule) which dissipated a power of 15 nW when the optical transition was saturated.[9] More precisely, a DNA-based construct was used, in which two of these chromophores were linked together. Another problem that had to be solved was that the change in refraction index induced by the heat release after light absorption is relatively small. The so-called transduction efficiency was increased by using glycerol (instead of water), a solvent in which heat conduction is weak. In addition, relatively high probe powers in the 100 mW range have been used, which helped to significantly reduce photon noise from the light beam.

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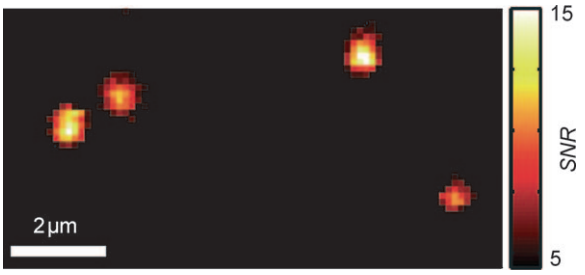

Figure 1. Photothermal image of four individual molecules each consisting of two chromophores (see text). Heating (pump) power at 514 nm: 5.1 mW; probe power at 800 nm: 84 mW (reproduced from Ref. [9]).

In Figure 1 a photothermal image of single molecules which were deposited on a substrate and submerged in glycerol is shown. The clearly visible spots appeared with a good SNR of 10 (in a detection bandwidth of approximately 3 Hz). Interestingly, in the absence of oxygen most such spots persisted for very long times, up to an hour. It was found that the average number of photons absorbed before irreversible photobleaching was 10^{11} under oxygen-saturated conditions and more than 10^{12} in the absence of oxygen. This photostability is very high compared to typical organic fluorophores and was attributed to the fast radiationless decay rate, which leads to a quick depletion of the excited singlet state and presumably a very small probability for intersystem crossing into the triplet state. Because of their long lifetime both of these states are more prone to photochemistry. From the change in signal after photobleaching, Gaiduk et al. could estimate the corresponding change in absorption cross section. The average value of 4.1×10^{-16} cm^2 (4.1 Å^2) was in good agreement with expectations derived from the bulk absorption spectrum.

The main obstacles impeding single-molecule absorption measurements under ambient conditions have been discussed above. Yet, soon after publication of the work just described, two other groups have succeeded in reaching shot-noise limited single-molecule sensitivity at room temperature.[12,13] The Sandoghdar group has reported single-molecule detection with a standard transmission type experiment.[12] This achievement has been accomplished because they could substantially suppress the noise sources arising from intensity fluctuations of the laser beam and scattering from the substrate. To account for residual background, the modulation-free detection scheme relied on measurement of the differential transmission before and after photobleaching of the organic dye molecules. The Xie group has applied a nonlinear optical technique, ground-state depletion microscopy, to demonstrate single-molecule absorption contrast.[13] In this approach two slightly different laser wavelengths (pump and probe beams) are irradiated into the molecular absorption band. The pump beam is intensity modulated at approximately 2 MHz. This modulation is transferred to the probe beam which senses the changes in transmission arising from depletion of the ground state at the modulation frequency. As in photothermal contrast, scattering from the sample does not contribute to the signal.

Suddenly having several sophisticated techniques at hand which allow single-molecule absorption detection under ambient conditions, the question arises what are the prospects of these breakthroughs? To date all the reports have been mainly proof of principle. In principle, the large number of non-fluorescing molecules (including bio-molecules) is now available for single-molecule studies, provided that the signal strength is sufficient. Hence, for future applications it appears to be vital to further improve the methodologies, which will be a challenging task, since they already operate at the shot-noise limit. In principle, integration times can be increased which, however, will limit the time resolution of the experiments. In case of the photothermal approach better thermal isolation from the substrate is going to improve the signal. From a material point of view the absorption cross sections can be enhanced through extended π-conjugation, shifting the absorption into the near-infrared, where fluorescence quantum yields anyway are often small. Another option to increase the effective absorption cross section may be the use of optical antennas, by which the light–matter interaction can be enhanced. A fascinating perspective would be to measure a single-molecule absorption spectrum. The results then could be compared to fluorescence data opening a window into the entire photophysics of a single molecule. An assessment of absorption and emission would contribute to unraveling the mechanisms of photoblinking and photobleaching of single molecules, which are currently poorly understood.[14] Recording of the full absorption spectrum will be a tremendous task, starting with the selection of a suitable light source up to proper beam alignment for the two-color techniques, because the color of one beam has to be tuned. Importantly, as has been demonstrated by the Orrit group, chromophores with sufficient photostability for long-term measurements can be found. Besides these more fundamental photophysical issues, it will be interesting to watch whether the techniques will allow single-molecule absorption detection in biological media, which are far from the ideal conditions tested to date. In the end, the crucial question will be whether single-molecule absorption can compete with fluorescence based techniques. Honestly, this author has some doubts, but is ready to be convinced otherwise.

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