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Single-Molecule Sensitivity in Optical Absorption at Room Temperature

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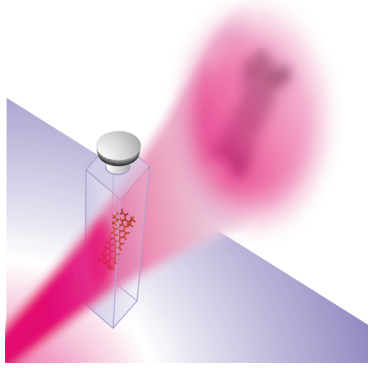
ABSTRACT Sensitive detection of condensed matter is of utmost importance in fundamental research as well as cutting-edge applications such as molecular analytics and diagnostics. Until very recently, all existing methods for the detection of single molecules at room temperature have required highly efficient fluorophores. Here, we demonstrate, for the first time, that single molecules can also be detected via standard modulation-free absorption measurements. Our work extends single-molecule detection to a huge class of materials that absorb light but do not fluoresce efficiently.

SECTION Kinetics, Spectroscopy



Absorption measurements are common place in the laboratory because they provide a convenient and label-free means for characterizing an unknown material via its absorption spectrum. In a typical experiment, a macroscopic sample is held in a beam of light and attenuates it according to the Beer−Lambert law. To perform such a measurement at the single-molecule level, one would need to compare the power of the light beam with and without a single molecule in its path, which can be achieved by translating the molecule in and out of the incident light. Despite its apparent simplicity, such an approach has proved unsuccessful over the past decades. As a result, alternative techniques based on cryogenic line narrowing,[1] Sagnac interferometry,[2] thin-film interferometry,[3] the photothermal effect,[4] stimulated emission,[5] and high-frequency modulation[6] have been explored for sensitive detection of optical absorption.

The major difficulty in performing single-molecule absorption detection at room temperature stems from the discrepancy between the absorption cross section ($\sim 10^{-15}$−10^{-16} cm^2) and the minimum size of a light beam dictated by the diffraction limit ($\sim 10^{-9}$ cm^2). A simple estimate suggests that to detect the expected absorption effect directly, one would have to suppress any intensity fluctuations in the detected light beam below the parts-per-million level. However, laser intensity fluctuations are invariably orders of magnitude larger than the desired signal on the time scale of translating the molecule. Furthermore, any background scattering caused by sample inhomogeneities or nearby molecules easily modifies the signal, overwhelming the tiny absorption signature. Here, we show that both of these noise sources can be successfully suppressed by combining balanced detection and an index-matched sample geometry. We thus achieve single-molecule absorption sensitivity in a simple and direct manner, which can be readily implemented in various applications.

Our experimental setup resembles a single-molecule variant of the standard Beer−Lambert type experiment, as depicted in Figure 1a. We split the linearly polarized output of a fiber-coupled helium−neon laser at a wavelength of 633 nm into probe and reference beams. Fiber-coupling was essential to reduce beam-pointing fluctuations as well as optimizing the mode to achieve the smallest possible beam area. The probe was injected into a home-built inverted microscope equipped with a closed-loop piezoelectric stage for sample scanning. Two matched oil-immersion microscope objectives with numerical apertures of 1.4 focused the incident laser beam onto the sample and collected it in transmission. Probe and reference beams were focused onto a balanced photodetector (Nirvana, New Focus) and adjusted to an intensity ratio of 1:2, respectively. In this way, we were able to reduce laser intensity fluctuations by more than 50 dB down to the shot noise limit. Probe and reference beam intensities then only differed by the presence or absence of a molecule in the focus as the sample was translated.

Achieving shot-noise-limited detection below the parts-per-million level is a nontrivial task, even with sophisticated laser noise cancellation systems such as balanced photoreceivers, because a typical level of laser intensity noise has to be reduced by about 5 orders of magnitude to ensure single-molecule sensitivity in absorption. In our particular case, where the log output of the Nirvana detector at maximum loop bandwidth was used, shot-noise-limited fluctuations in the electronic output of the detector were much too small to be detected by a standard data acquisition card. To understand this, it is useful to consider our experimental conditions; 100 μW incident power is accompanied by shot-noise-induced photocurrent fluctuations of about 10^{-10} A rms for a measurement bandwidth of 1 kHz and a photodiode responsivity of 0.45 A/W. Taking into account the shot noise of the reference (power P_{ref}) and the signal (power P_{sig}) beams and that the Nirvana output corresponds to $V = -\ln(P_{ref}/P_{sig} - 1)$, the resulting fluctuations amount to about 7×10^{-6} V.

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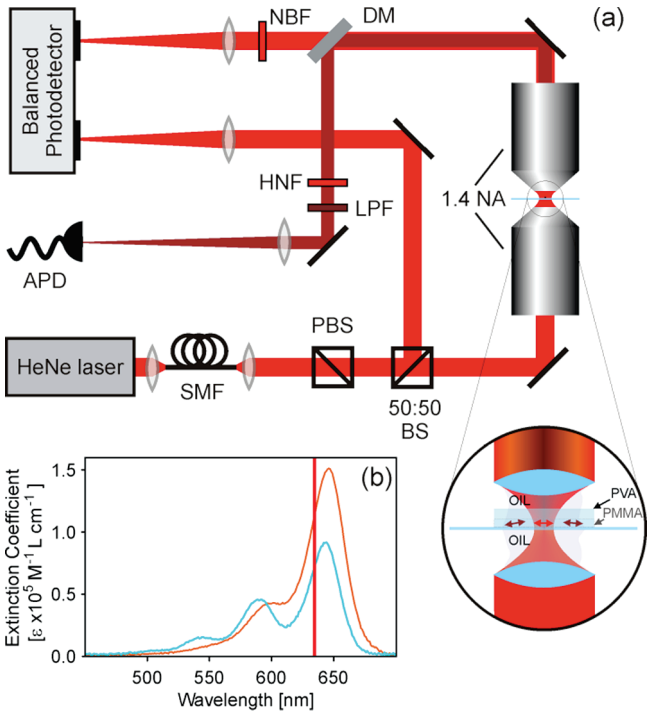

Figure 1. (a) Experimental setup. He−Ne: helium−neon; SMF: single-mode polarization-maintaining fiber; PBS: polarizing beam splitter; BS: beam splitter; APD: Avalanche photodiode; HNF: holographic notch filter; LPF: long pass filter; DM: dichroic mirror; NBF: narrow band filter; NA: numerical aperture. Inset: sample construct. The single molecules studied were either terrylene diimide (TDI) or Atto647N. TDI was embedded in an 80 nm thin layer of polymethylmethacrylate (PMMA) and covered by a 1 μm layer of polyvinyl alcohol (PVA) for protection from microscope immersion medium. Atto647N was spin-cast directly onto a microscope cover glass and then protected by a 1 μm PVA layer. (b) Ensemble absorption spectra of TDI (blue) in toluene and Atto647N (red) in water. The laser excitation wavelength at 633 nm is indicated for clarity. Note that these spectra may differ for the polymer hosts used in this experiment.

To be able to measure such small signals, we have used an additional voltage amplifier (Stanford Research Systems, SR560), which is impedance-matched with the Nirvana output. The amplification was maximized to 5×10^3 while avoiding saturation of the amplifier input (3 V peak-to-peak) during sample scanning. In addition, we enabled a built-in 3 kHz low-pass filter at 12 dB to reject high-frequency noise and operated in the low-noise mode. Given that the amplifier noise of 4 nV/Hz$^{1/2}$ at 1 kHz was much smaller than the shot-noise-induced voltage fluctuations at the output of the detector and that the resulting voltages were on the order of 35 mV, we could reliably measure the shot noise of the signal beam even at low incident powers with standard data acquisition boards (National Instruments BNC-6229). We emphasize that we have detected single molecules with incident powers down to 10 μW (see Figure 4). We remark that the same performance could also be obtained for powers below the μW range, where the common-mode rejection ratio of the detector decreases, if the laser intensity is stabilized externally.

To demonstrate single-molecule sensitivity in a robust and convincing fashion, we chose fluorescent dye molecules as absorbers because their well-established properties such as photoblinking and single-step photobleaching allowed us to identify single molecules unambiguously. Thus, we simultaneously recorded the absorption signal with any fluorescence emitted by the sample. The two detection paths were separated by a dichroic mirror and additional long-pass filters. A narrow-band filter was inserted in the probe path to reject any residual fluorescence.

Background scattering was minimized using a completely index matched geometry. As demonstrated in interferometric scattering detection, the magnitude of scattering background caused by sample imperfections such as surface roughness relative to a resonant signal can be reduced significantly by index matching.[7,8] Our samples therefore consisted of a standard microscope cover glass coated by a thin polymer layer containing a nanomolar concentration of the molecular dyes terrylene diimide (TDI)[9] or Atto647N (Atto-tec, GmbH), both of which absorb efficiently at 633 nm (see Figure 1b). The refractive indices of the glass (1.52) and the polymer layer (1.49−1.51) closely resembled that of standard microscope immersion oil (1.518), generating an almost perfectly index matched geometry. In such a way, optical sample roughness could be reduced to 10^{-6} rms.

Figure 2a depicts a fluorescence image obtained by raster scanning a TDI sample across the diffraction-limited focus spot of the laser. We observed significant variations in fluorescence intensity and spatial features caused by the interaction of linearly polarized light with randomly oriented dipole moments.[10] To select the optimized projection of the illumination polarization onto the absorption dipole moment of the molecules under study, we performed our detailed measurements only on the most intense emitters for a constant incident power. After choosing a molecule, we recorded repeated lateral line scans across the maximum of its fluorescence spot (Figure 2b). The resulting fluorescence traces invariably exhibited fluorescence blinking and one-step photobleaching, which prove that the absorption signal stems from single molecules.

Simultaneously acquired absorption maps were dominated by shot-noise-induced fluctuations of the laser intensity and residual interferometric scattering. Nevertheless, as shown in Figure 2c, a distinctive change in the differential transmission could be observed after photobleaching. Figure 2d displays the fluorescence intensity and differential transmission averaged over 200 nm about the center of the molecule for each line in Figure 2b and c. The resulting time traces revealed a stepwise change of about 3×10^{-6} in the probe transmission occurring simultaneously with photobleaching.

The reproducibility of the features in Figure 2c after photobleaching illustrates the residual contribution of sample surface roughness or slight variations in the index of refraction. The orange curve in Figure 2e presents a quantitative measure of these signal fluctuations on the order of 3×10^{-6} root-mean-square (rms). Here, we have averaged successive line scans and thereby reduced the intensity fluctuations induced by the shot noise. The black trace in Figure 2e shows that these background fluctuations could be suppressed further by an order of magnitude to 5.3×10^{-7} rms if two such averages were subtracted. The remaining fluctuations agree well with the expected shot-noise limit of 7×10^{-7}.

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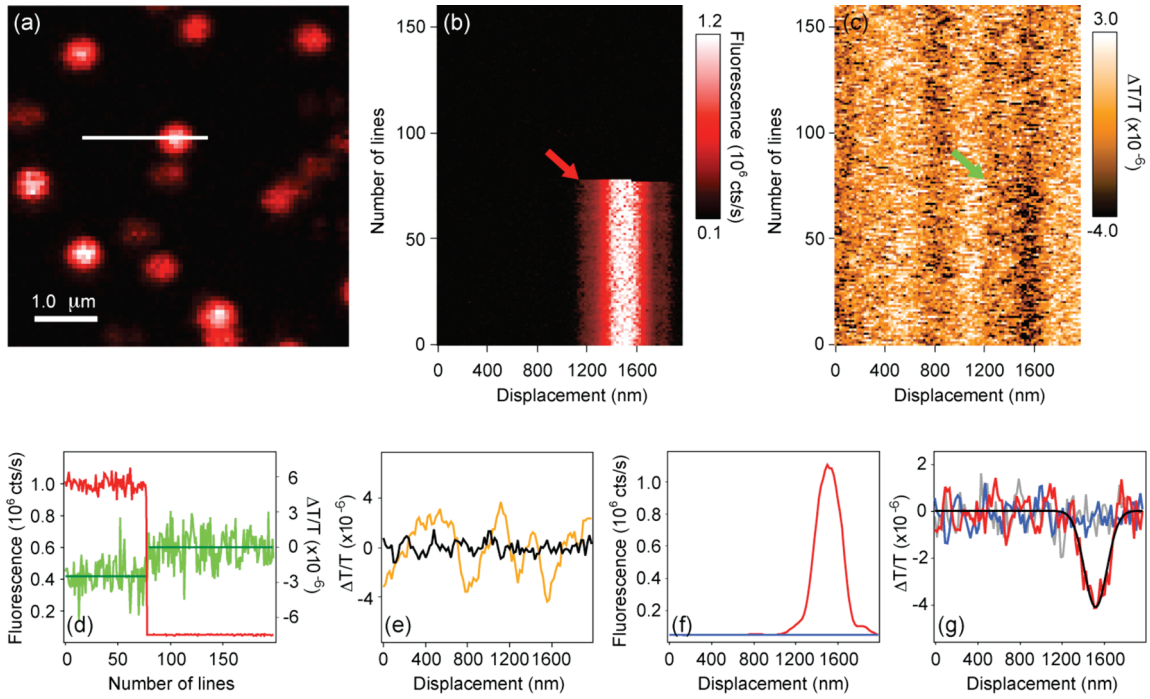

Figure 2. (a) Fluorescence raster scan of TDI molecules in PMMA. (b) Fluorescence image constructed from repeated lateral scans of the molecule marked in (a). (c) Corresponding differential transmission image. Acquisition parameters are 100 μW incident power, 1 ms pixel dwell time, and 20 nm/pixel step size. Other features in this image indicate residual background inhomogeneities, which are eliminated upon subtraction (see, e.g., (g)). A low order polynomial baseline was subtracted for each line to remove low frequency fluctuations ($<$ 10 Hz). The arrows indicate the bleaching event. (d) Fluorescence (red) and absorption (green) time traces from (b) and (c) after averaging over 200 nm about the center of the molecule. (e) Lateral differential transmission scans averaged over 80 consecutive lines (orange) and subtraction of two such consecutive averages (black) in the absence of any molecule. (f) Averaged fluorescence line scans for the molecule in (b) before (red) and after (blue) photobleaching. (g) Corresponding differential transmission scans. Subtraction of the averaged scan in the off state from the averaged scan in the on state reveals a clear transmission dip (red). When both averages are chosen from the off (blue) or on (gray) states, no dip is visible.

Having quantified and minimized the fluctuations of the signal transmitted through the sample, we can now subtract two line scan averages chosen before and after photobleaching (see the arrows in Figure 2b and c). Figure 2f and g depicts the results for the fluorescence and transmission signals, respectively. The averaged absorption scans during molecule emission (red trace) exhibit a clear transmission dip with a magnitude of $\Delta T/T \approx 4 \times 10^{-6}$ at the same lateral position as the fluorescence peak. We emphasize that, as shown by the blue and gray traces in Figure 2g, no such dip could be observed if both averages were chosen from the regions before or after bleaching. The observation of a dip in the red curve of Figure 2g, therefore, confirms that the absorption cross section is diminished upon photobleaching. We note that the above-mentioned averaging and subtraction procedure allowed us to detect the absorption signal of single molecules even when they were not readily visible in the raw data, as was the case in Figure 2c and d. Importantly, repeated lateral scanning rather than full imaging allowed us to reduce absorption of incident light by the molecule even when the incident beam and the molecule were not aligned perfectly. In this way, we could optimize the duty cycle of light exposure and absorption detection and reduce the shot-noise-induced background fluctuations before photobleaching.

In some cases, our selection for maximum brightness resulted in two neighboring molecules in the focus. The fluorescence line image in Figure 3a and a time trace of the spatially averaged fluorescence intensity shown in Figure 3b display two clear bleaching steps. The simultaneously acquired averaged absorption scans also revealed two dips in the differential transmission of different magnitudes for two molecules (6.5×10^{-6}) and one molecule (3.6×10^{-6}). This constitutes further and definite proof of the single-molecule sensitivity achieved by our setup.

Our ability to probe the molecule even in the absence of fluorescence allowed us to investigate the absorptive properties of individual molecules during fluorescence blinking, as demonstrated in Figure 3d. The red and blue curves in Figure 3e depict fluorescence line scans during the blinking on and off states, respectively. The red trace in Figure 3f plots the line scan obtained by subtracting averages of the transmission signal when the molecule was emitting from those when it was intermittently switched off, revealing a clear transmission dip at the same lateral position as the fluorescence maximum. Subtraction of averages during the off state does not show any feature at the position of the molecule, as confirmed by the blue trace in Figure 3f. The total lack of any residual absorption at the excitation wavelength strongly suggests that in TDI, blinking is due to a transition to a dark triplet state without an appreciable absorption cross section at 633 nm. This is in contrast to our previous report on semiconductor nanocrystals, where the extinction cross section

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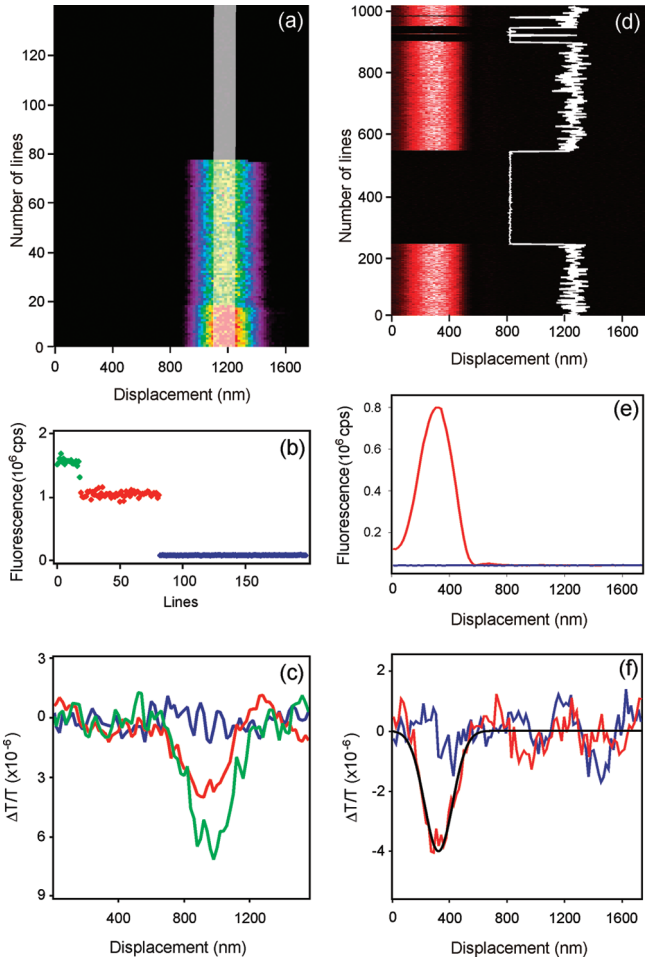

Figure 3. (a) Fluorescence line scan image for two close-lying molecules. (b) Averaged fluorescence intensity for the molecules in (a). (c) Corresponding differential transmission scans for two (green), one (red), and no (blue) emitting molecule. (d) Fluorescence line scan image for a molecule exhibiting long-term fluorescence intermittency. The corresponding fluorescence time trace relative to a line cut is overlaid in white. (e) Averaged fluorescence line scans when the molecule is in the on and off states. (f) Corresponding differential transmission scans for the molecule in the on (red) and off (blue) states.

remained unchanged during the off state. In that system, blinking is presumably caused by quenching rather than a transition to a different quantum state.[11]

A distinct advantage of absorption over fluorescence detection is its insensitivity to the emissive properties of the molecule under study. To demonstrate this phenomenon experimentally, we examined Atto647N molecules, which have been proposed to be quenched in polyvinyl alcohol (PVA) via an electron-transfer process.[12] Figure 4a confirms that the fluorescence of such a sample is extremely weak and undergoes intermittent emission. The blue curves of Figure 4b and c display the averages of successive lines after photobleaching for fluorescence and absorption, respectively, while the red traces in these figures plot the subtraction of averages before and after photobleaching. We again find an absorption dip at the same position as the fluorescence maximum.

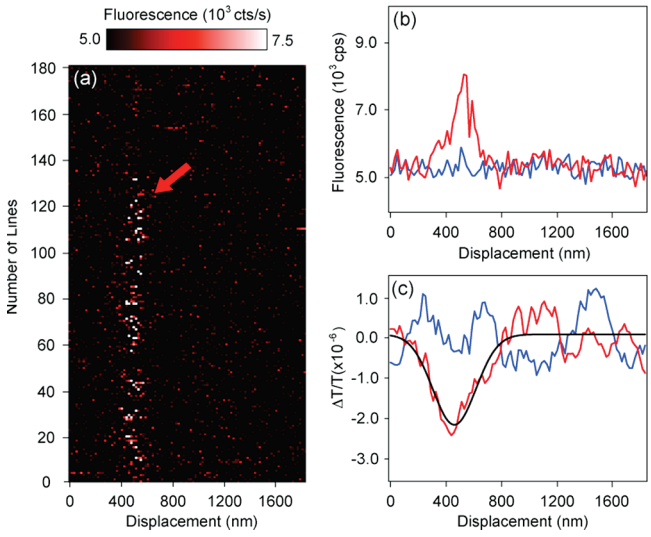

Figure 4. (a) Fluorescence line scan image for a single Atto647N molecule covered by PVA. The incident power has been reduced to 10 μW to prevent rapid photobleaching. The red arrow suggests the bleaching event. (b) Averaged fluorescence intensities before (red) and after (blue) photobleaching. (c) Corresponding differential transmission scans. The black curve represents a Gaussian fit to the observed transmission dip.

We note that although the emission level here is more than 30 times weaker than that of TDI, the absorption signal of a single Atto647N molecule embedded in PVA is still comparable to that of TDI. The lower value of the recorded absorption dip can be explained by our inability to confidently select well-aligned molecules as well as the inevitable inclusion of blinking off times in our averaged traces. The signal-to-noise ratio is inferior because of lower count rates and, thus, higher shot noise. Observation of the different behaviors of the two molecules TDI and Atto647N in PVA during their off states demonstrates one of the promises of our work. We envisage that simultaneous single-molecule absorption and fluorescence spectroscopy will allow one to distinguish between various mechanisms such as fluorescence quenching and transition to nonabsorbing states, while fluorescence detection alone is incapable of identifying the cause for its own absence.

The goal of our current proof-of-principle work has been to demonstrate that, contrary to an often-communicated general belief,[4] it is possible to reach single-molecule sensitivity in direct modulation-free absorption spectroscopy. Here, we have relied on photobleaching or photoblinking to prove single-molecule sensitivity and to separate the transmission signal of interest from the background. However, it is also possible to account for the background by performing measurements at different wavelengths and thus modulating the signal according to the absorption spectrum of the species at hand.[7] This approach would not only allow the detection of single molecules in their native "live" state, but it would also initiate the exciting era of single-molecule absorption spectroscopy. The latter is of fundamental interest because it provides insight into molecule−host interactions for a wide range of materials that absorb light but do not fluoresce. Furthermore, absorption spectroscopy opens doors to single-molecule studies

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in the infrared, where fluorescence detection has been hampered by noisy single-photon detectors.

In closing, it is instructive to compare our direct transmission approach with the photothermal effect, which has also succeeded in reaching single-molecule sensitivity during our publication process.[4,13] In photothermal detection, the change in the refractive index (n) of the surrounding of the molecule is detected via interferometry with a second laser beam. This technique relies on the use of an appropriate matrix material with a sufficiently large dn/dT to detect a small change of temperature (T) caused by the energy dissipation of a single molecule. In a standard transmission measurement, however, the dissipated energy is measured directly via homodyne interferometry,[14] making this method intrinsically more sensitive, simpler, and more universally applicable. The signal-to-noise ratio of the recent data reported by both approaches is currently around 10, although our measurements were performed at much lower incident power and integration times. All of these developments promise to open new doors in nano-optical studies in the near future.

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