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Low temperature single molecule spectroscopy using vibronic excitation and dispersed fluorescence detection

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We demonstrate vibronic excitation combined with spectrally resolved zero phonon line detection of single terrylenediimide (TDI) molecules in both the Shpol'skii matrix hexadecane and the polymer PMMA at cryogenic temperatures. Spectral jumps as large as 80 cm^{-1} are recorded with a 1 s time resolution. This technique does not require a weak electron–phonon coupling, and promises applications in spectroscopy of a wide-range of single nanostructures in amorphous hosts including fluorescing proteins. Vibronic excitation is also used to determine vibrational spectra of the excited state of single TDI molecules. © *2003 American Institute of Physics.* [DOI: 10.1063/1.1582845]

Since its first achievement more than a decade ago, single molecule detection has become a flourishing field of research encompassing areas as diverse as quantum optics, polymer physics, and molecular biology.[1,2] Experiments at cryogenic temperatures yield unprecedented insights into chromophore photophysics and into the coupling of chromophores to the surrounding matrix.[3] In the latter case, the absolute frequencies of the electronic transitions of single molecules serve as exquisitely sensitive spectroscopic reporters for the matrix dynamics. Investigations of this kind are especially important for gaining a better understanding of the chromophore matrix interactions in dye doped polymers and photoactive proteins. The amorphous character of such systems however leads to spectral dynamics within a wide spectral range which cannot be followed with established techniques.

In the earliest low temperature experiments, minute changes in the absolute position of the purely electronic zero-phonon line (ZPL) absorption of a single molecule were studied by scanning the frequency of an exciting narrow bandwidth laser and collecting the integral Stokes shifted fluorescence.[4] This technique has also been used to measure the dispersed fluorescence emission of single molecules.[5] A major problem of exciting the ZPL, however, is that only a few stable systems can be studied. Because the absorbing ZPL is so narrow, already small spectral jumps of the molecular absorption lead to a loss of the excitation. Hence this technique can only be used to detect spectral jumps smaller than 30 GHz. Only few attempts to observe single molecules without excitation of a narrow ZPL have been reported. The first class of experiments comprises photosynthetic pigments.[6,7] In this case the strong electron–phonon coupling of the chromophores results in broad absorption bands. A different approach was taken by Plakhotnik and coworkers who reported the excitation of a vibrational line of terrylene molecules in a naphthalene crystal without a definite proof for single molecule detection.[8] The high photostability of their system prompted the authors to detect vibrational lines of single molecules by saturation spectroscopy, which is applicable to only exceptionally photostable systems.[9] At room temperature, rapid collection of single molecule emission spectra is possible at the expense of emission bands broadened to tens of nm which brings a severe loss of information.[10] Room temperature experiments further suffer from diminished photostabilities.

The technique presented here relies on the combination of confocal low temperature microscopy with vibronic sideband excitation and spectrally resolved ZPL detection. At cryogenic temperatures, due to their short lifetimes (~1–10 ps), the excited vibrational states are generally very broad. By contrast, the width of the purely electronic ZPL is typically determined by the long radiative lifetimes (~several ns) of the excited electronic states. The usable absorption width is further increased to tens of cm^{-1} in systems with a strong electron–phonon coupling due to intense phonon sidebands. Spectral jumps of the molecular absorption which remain within the width of the absorption band can therefore be followed by detecting the spectrally resolved ZPL emission. ZPL detection is advantageous as it offers both, high sensitivity and high spectral selectivity in that a large part of the excitation energy is emitted in the spectrally narrow ZPL. As we demonstrate in this paper, vibronic excitation is applicable to a wide range of systems including amorphous systems.

Our experiments are performed with a home-built confocal microscope working at temperatures of 2 to 5 K. A single mode dye-laser is reflected off a dichroic mirror and a galvo optic mirror scanner, directed through a telecentric system and focused onto the sample with an aspherical lens (NA 0.55). Excitation intensities between 50 μW–5 mW are used due to relatively low absorption cross sections of vibronic bands. Redshifted emission light is collected with the same lens, transmitted through the dichroic mirror, filtered with a notch filter, and focused through a 300 μm diameter pinhole. Subsequently the fluorescence emission is dispersed with a 46 cm monochromator before detection with a CCD camera.

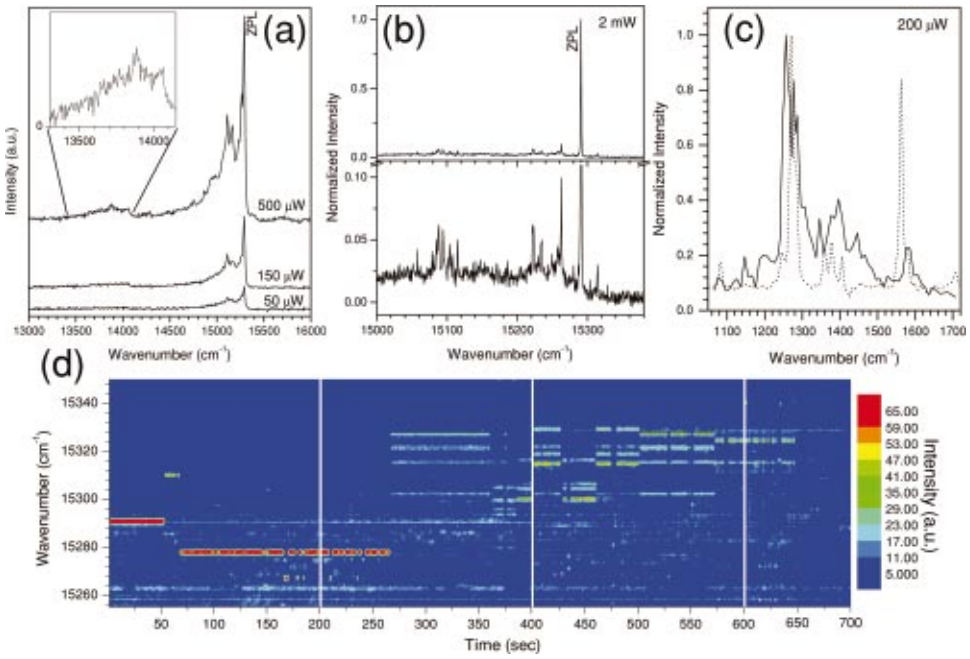

FIG. 1. (Color) (a) Low resolution and (b) high resolution emission spectra of a single TDI molecule in HD. Two different magnifications are plotted in (b) for clarity. (c) Solid line: Vibronic fluorescence excitation spectrum, x-axis is referenced to ZPL emission at 15 291 cm^{-1}. Dotted line: Raman spectrum of TDI in $CHCl_3$. (d) High resolution emission spectra with 5 mW excitation intensity 1254 cm^{-1} blueshifted from the ZPL. The four consecutive segments are separated by a few seconds of unexcited period.

The spectral resolution of our setup is 35 GHz. We examined terrylenediimide (TDI) molecules[11] highly diluted in hexadecane (HD) or polymethylmetacrylate (PMMA) matrices. Hexadecane forms a Shpol'skii matrix which provides an ordered, partly crystalline surrounding for the TDI molecules, whereas the polymer PMMA is an amorphous host.

We observe spectrally stable ZPL emissions in the crystalline HD host under vibronic excitation. Emission and absorption characteristics of a TDI molecule in HD are shown in Figs. 1(a)–1(c). Figures 1(a) and 1(b) are taken using laser excitation blueshifted from the ZPL by 1254 cm^{-1}. Absorption at this energy is relatively efficient as discussed in the next paragraph. Vibronic emission lines observed in Fig. 1(a) are in agreement with values reported in Ref. 11 for TDI in a polyethylene matrix. Figure 1(b) is a zoom into the region near the sharp ZPL at 15 291 cm^{-1}. In this high resolution spectrum, the phonon sideband of the ZPL is seen as the pedestal at the low energy side together with some other sharp emission lines. Sharp emission lines in Fig. 1(b) are due to both vibronic emission of the specific molecule and ZPLs of some other molecules located in the vicinity. The resolution limited ZPL emission in Fig. 1(b) indicates that the molecule does not show spectral diffusion larger than 35 GHz within the 200 s integration time.

Figure 1(c) shows the vibronic fluorescence excitation spectrum of the ZPL in Figs. 1(a)–1(b) where six vibrational resonances are clearly identified. The observed vibrational frequencies compare well to the Raman spectrum of TDI dissolved in $CHCl_3$ which is plotted as the dotted curve. Two explanations for the deviations between the bulk Raman spectrum and the vibronic excitation spectra seem likely. The first is the fact that the Raman spectra refer to vibrational levels in the electronic ground state while the vibronic excitation spectra monitor the vibrations in the first excited electronic state. A second influence is certainly the interaction of the molecule with the local environment which gives rise to vibrational heterogeneities.[5] Data for three other molecules together with this molecule and the Raman spectrum are collected in Table I. For molecule 3, spectral jumps of the ZPL as shown in Fig. 1(d) were observed as an unambiguous signature of single molecule detection, while molecules 1 and 2 showed similar resolution limited signal intensities that were spatially highly localized, but no spectral jumps.

The comparison of the vibronic fluorescence emission band in the inset of Fig. 1(a) with the fluorescence excitation spectrum in Fig. 1(c) shows that the purely vibronic bands are weaker and have more intense phonon side bands in emission compared to absorption. This can be explained by anharmonic potentials for vibrations and phonons, or by assuming different one-phonon spectral distribution functions in the ground and excited states using harmonic potentials.[12]

In order to prove that the signal observed in Figs. 1(a)–1(c) indeed originates from a single molecule, we raised the excitation intensity to 5 mW and recorded consecutive high resolution emission spectra taken with 1 s exposure times. As depicted in Fig. 1(d), after 54 seconds at the original spectral position, a first jump of 19 cm^{-1} in the spectral position of the ZPL is observed. It is subsequently followed by a series of further jumps. We attribute the pronounced increase of observed jump positions and frequencies after 270 seconds to the thermal activation of local degrees of freedom by laser induced heating. The observed jumps are a clear indication of single molecule observation. In the spectral trace depicted

TABLE I. Vibronic absorption resonances of four different TDI molecules compared to the bulk Raman spectrum. The intense absorption at ~1285 cm^{-1} only appears as a shoulder in the Raman spectrum.

	Vibrational frequencies (cm^{-1})					
Raman (bulk)	1271	1284	1359	1378	1406	1563
Molecule in Fig. 1	1261	1281	1346	1391	1446	1581
Molecule 1	1269	1292	-	-	-	1597
Molecule 2	1264	1287	-	-	-	1597
Molecule 3	1265	1279	-	-	-	1593

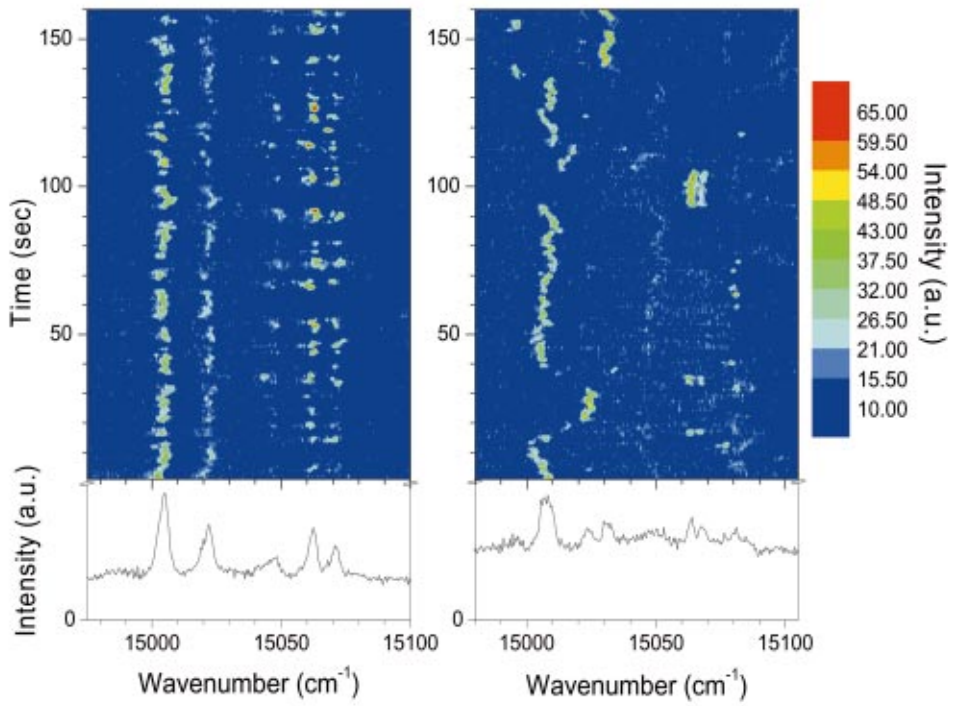

FIG. 2. (Color) High resolution emission spectra of two TDI molecules in PMMA host. Excitation is at 16 498 cm^{-1} 3 mW (left) and 2 mW (right).

in Fig. 1(d), the spectral jumps occurred on a THz scale which is approximately three orders of magnitude larger than the expected ZPL linewidth. If the excitation of the molecule via its ZPL absorption would have been used, the molecule would not have been excited any more after the first jump. This would have been interpreted as photobleaching or blinking, depending on whether the new spectral position was stable without further illumination.

Figure 2 shows a sequence of vibronically excited emission spectra of two single TDI molecules in the PMMA host taken with a time resolution of 1 s. As expected for an amorphous matrix, we find that under comparable excitation conditions, the probability of spectral jumps of TDI is much higher in PMMA than in HD. This behavior concerns large spectral jumps on the order of 80 cm^{-1}, but also small spectral changes as exhibited by the jitter in the ZPL spectra depicted in Fig. 2. The variety of spectral diffusion behaviors as seen in Fig. 2 can be analyzed on basis of the established two-level system model.[4] In contrast to the TDI/HD sample, in TDI/PMMA we find resolution limited ZPL only in some of the individual spectra, while in most cases linewidths of up to 300 GHz are detected.

Large spectral jumps have been detected in single light harvesting complexes by scanning the excitation wavelength.[13] In contrast to excitation spectroscopy in which the spectral data are recorded sequentially, emission spectroscopy with dispersion on a CCD camera offers the advantage of parallel data acquisition. Apart from the increased time resolution (one order of magnitude), in emission spectroscopy the molecule is permanently excited and all spectral positions within the detection window are monitored at all instances.

The limits of our method for studies of chromophores in amorphous solids are given by the strength of the electron–phonon coupling, the lifetime of the purely electronic zero phonon transition and spectral diffusion. Electron–phonon coupling determines the intensity ratio between the ZPL emission and the phonon sideband emission which in the low temperature limit scales as $\exp(-S)$, where S is the Huang–Rhys factor. It is obvious that strong electron–phonon coupling will severely decrease the intensity of the ZPL. However provided that the lifetime of the purely electronic ZPL is long and that spectral diffusion during the integration time is limited, the width of the ZPL will be much narrower than that of the phonon sideband. Under these circumstances, the ZPL will appear as a detectable sharp line next to a broad fluorescence background. This expectation is further supported by the burning of 3 GHz wide spectral holes in bands of the chromophores in photosystem I which exhibit strong electron–phonon couplings.[14,15]

We have shown that the combination of confocal microscopy, vibronic side band excitation, and dispersed detection of ZPL emission can be used to efficiently detect single molecules at cryogenic temperatures. This technique opens access to high resolution spectroscopy of a wide variety of molecular systems as it does not necessitate a spectrally stable ZPL. Therefore it should also lend itself to studies of fluorescing proteins. The 1 s temporal resolution of our setup should easily be improved by an order of magnitude with better collection efficiency. Newly available high numerical aperture low temperature microscope objectives, parabolic mirrors, or solid immersion lenses can be used for this purpose. We have also demonstrated how ZPL detection in emission can be used to record vibronic excitation spectra of the excited state of single TDI molecules in HD.

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