

# High photo-stability of single molecules in an organic crystal at room temperature observed by scanning confocal optical microscopy

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Single molecules have been observed in a molecular crystal at room temperature using scanning confocal fluorescence microscopy. Individual terylene molecules in a *p*-terphenyl crystal show high photo-stability with a corresponding ensemble-averaged photo-destruction quantum efficiency lower than  $1.2 \times 10^{-8}$ . On average, with a detection efficiency of 6.5%, a stable fluorescence signal of  $10^5$  photons  $s^{-1}$  could be detected from single molecules during 1 min of continuous photo-excitation. Both irreversible and reversible abrupt fluorescence intensity jumps to the background have been observed. The experimental data indicate that diffusing quenchers at low concentration in the crystal contribute to fluorescence bleaching of single molecules. All molecular fluorescence signals follow a typical power saturation law with a mean saturation count rate of  $4.3 \times 10^5$  photons  $s^{-1}$ . The large photo-stability allows for long illumination times and high emission rates of single molecules trapped in a crystal at room temperature. A potential application as single organic quantum light sources under ambient conditions is conceivable.

## 1. Introduction

In the past few years the detection of single molecules has attracted considerable attention for basic and applied research in the fields of physics, chemistry and biology. The combination of microscopy and spectroscopy of individual molecules represents a powerful tool to probe various environmental conditions at a microscopic scale [1]. In contrast to ensemble measurements, single molecule experiments allow for the determination of distributions of parameters. Single molecules have been investigated at room temperature in water streams [2], droplets [3], gels [4], surfaces [5–7], thin polymer films [8, 9] and fluid lipid membranes [10]. Individual molecules have been also used as nanometric laboratories for single particle quantum optics [11, 12].

A molecule emits light for a time which is limited by photo-bleaching (i.e., a transition to a long lived dark state) provided that its residence time in the excitation focus is long enough. Thus photo-bleaching imposes an upper limit for the number of photons detectable from a single molecule. The photo-destruction quantum efficiency has been reported to range approximately from  $10^{-7}$  to  $10^{-5}$  in various systems [4, 7, 10, 13]. For experiments and applications at room temperature, it is of great importance to optimize the photo-stability by choosing appropriate experimental conditions. Good photo-stability makes it possible to maximize the

signal to noise ratio for a given experiment, since a single molecule can be studied at a high emission rate for a long period. In such a case, fascinating experiments, e.g., time-resolved spectroscopy and quantum optics experiments on single molecules in cavities, are greatly facilitated. In addition, near-field optical devices using single organic molecules as nanometric light sources can be envisaged. Here, we study the photo-stability of single terylene molecules in a *p*-terphenyl crystal. On average  $5.7 \times 10^6$  fluorescence photons were detected from individual molecules before photo-bleaching took place. This number of photons exceeds all previously reported values by more than one order of magnitude.

## 2. Experimental setup

The sample is an approximately  $10 \mu\text{m}$  thick sublimated crystal flake of *p*-terphenyl doped with terylene at a concentration of  $1.5 \times 10^{-10}$  mol  $l^{-1}$  as estimated from fluorescence confocal images. The flake is attached to a cover glass with a tiny amount of index matching oil. Previous low temperature studies indicate that terylene absorbs around 579 nm in the *p*-terphenyl crystal, having a fluorescence quantum yield close to unity [14].

Images of single molecules are recorded with a fluorescence scanning confocal optical microscope. The excitation beam (514 nm, argon ion laser) is passed through

a single mode fibre, collimated, directed to the entrance pupil of an oil immersion objective (Leica, NA = 1.3) by means of a dichroic mirror and focused to a diffraction limited spot inside the crystal. The fluorescence is collected by the same objective and directed to an avalanche photodiode (APD, EG&G, SPCM-AQ) passing the dichroic mirror. The residual scattering light is blocked by a 514 nm super notch filter and long pass colour filters. The small active area of the APD serves as a pinhole in the detection path. The detection efficiency of the setup is  $\eta \cong 0.065$ . Fluorescence images are recorded by raster scanning the sample through the fixed focus and counting the photons for each pixel within a given integration time.

### 3. Results and discussions

The spots visible in figure 1 are attributed to the fluorescence of single terylene molecules. The full width at half maximum of typical spots is approximately 350 nm. The peak count rates at an excitation intensity of  $900 \text{ kW cm}^{-2}$  range from  $1.6 \times 10^5 \text{ counts s}^{-1}$  to  $6.9 \times 10^5 \text{ counts s}^{-1}$  with a mean value of  $4.2 \times 10^5 \text{ counts s}^{-1}$ . With a pixel integration time of 1 ms, the signal to noise ratio is about 400 and the total time needed to record an image ( $256 \text{ pixels} \times 256 \text{ pixels}$ ) is 65 s. The signal to background ratio ranges from 3 to 12 for the most intensive peaks. At an integration time of 1 ms per pixel, several consecutive images of the same area are fully reproducible with all molecules showing up at the same positions in the images. Even if the integration time is increased to 20 ms per pixel, only a few

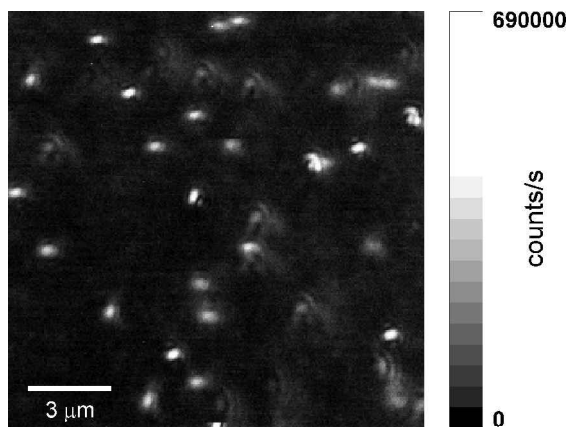


Figure 1. Fluorescence image ( $256 \times 256$  pixels) of a  $15.2 \mu\text{m} \times 15.2 \mu\text{m}$  sample depth section. Count rates are represented by a grey scale. Each molecule corresponds to a bright spot, which maps the excitation intensity distribution in the focus. The pixel integration time is 1 ms and the excitation flux is  $900 \text{ kW cm}^{-2}$ . The average number of photons recorded per molecule is  $15 \times 10^3$  for a total illumination time of approximately 35 ms.

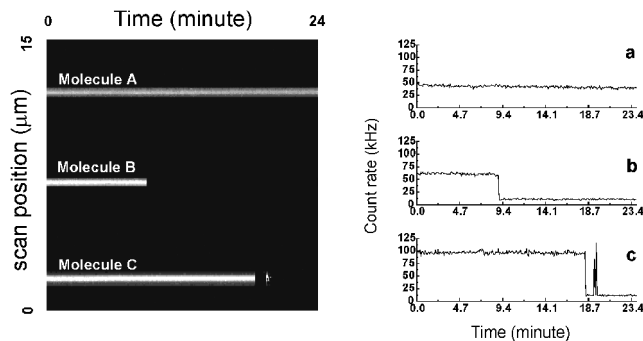


Figure 2. On the left is a line scan of  $15 \mu\text{m}$  length along the  $x$  direction at a constant  $y$  position as a function of time. The three detected molecules illustrate three characteristic behaviours. The righthand side shows fluorescence time traces obtained from a one pixel wide cut along the maximum of the fluorescence traces on the left.

per cent of the molecules are missing in the second of two consecutive images. Structures, characteristic for fast photo-bleaching such as stripes of a few pixels in length or half-moon shaped dots (see for instance [7]) are hardly ever found.

These observations reveal the high photo-stability of individual terylene molecules in a  $p$ -terphenyl crystal at room temperature. This new and fascinating feature has been studied by investigating the photo-stability of 41 single molecules at an excitation flux of  $225 \text{ kW cm}^{-2}$  and a pixel integration time of 22 ms. In figure 2, traces of repetitive line scans are shown for three molecules. Molecule A shows a stable fluorescence signal with a count rate of approximately  $5 \times 10^4 \text{ counts s}^{-1}$  during 256 line scans (trace a). For molecule B (trace b) the fluorescence intensity jumps irreversibly to the background count rate at  $t \cong 9 \text{ min}$ . In contrast, molecule C (trace c) shows reversible behaviour. After an intensity drop at  $t \cong 18 \text{ min}$ , the fluorescence recovers and fluctuates before it again drops irreversibly to the background. Similar fluctuations were observed for 15 out of 41 molecules in this experiment. Since the absorption spectra are relatively broad at room temperature, these fluctuations cannot be explained by spectral jumps out of resonance with the exciting laser frequency as previously observed for single molecules at liquid helium temperature [15]. Indeed, the largest spectral shifts, expected to be limited by the width of the inhomogeneous band of a few  $\text{cm}^{-1}$  typical for doped organic crystals, would be unobservable in our experiment because of the low spectral resolution ( $> 30 \text{ cm}^{-1}$ ). Furthermore, rotational jumps of the molecules (see, e.g., [16]), such that the dipole moment becomes accidentally oriented perpendicular to the light polarization, can be ruled out. From the crystal structure of the

closest shell surrounding the substitutional terylene molecule, the molecular orientation can be thought of being tightly fixed. However, strong changes either in the absorption cross-section  $\sigma$  and/or in the fluorescence quantum yield  $\phi_f$  may lead to the observed fluctuations [17]. We propose a mechanism that could explain the variations of the parameters  $\sigma$  and  $\phi_f$  namely fluorescence quenching by diffusing species, as discussed below. Note that recently photochemical bleaching has been demonstrated to produce energy traps in single light harvesting complexes [18] and single conjugated polymer molecules [19]. The fluorescence intensity fluctuations observed in these systems are attributed to the formation and decay of such traps which efficiently quench the fluorescence.

From the line scans we deduced the photo-stability quantitatively by determining the number  $N$  of detected photons emitted by each molecule which photo-bleached within the 256 line scans. The quantum efficiency for photo-destruction  $\phi_b$  was calculated from  $N$  according to  $\phi_b = (\eta\phi_f)/N$  where  $\eta$  is the detection efficiency and  $\phi_f$  denotes the fluorescence quantum yield. The unknown quantity  $\phi_f$  was set to 1. Therefore, the estimated values of  $\phi_b$  given in this paper represent upper limits. The histogram shown in figure 3(a) was obtained from 32 out of 41 molecules that photo-bleached during the 24 min total duration of the experiment. The histogram is truncated on the left side at  $\phi_{bt} \cong 2.6 \times 10^{-8}$ , corresponding to the smallest measured value of  $\phi_b$ . Fast photo-bleaching events occurring on a timescale shorter than  $\cong 20$  ms of photo-excitation are rarely observed and are not included in the statistics. No decrease in photo-stability is observed when the excitation flux is increased by a factor of 2. Consequently, the chemical stability depends only weakly on the local heating generated through non-radiative transitions of

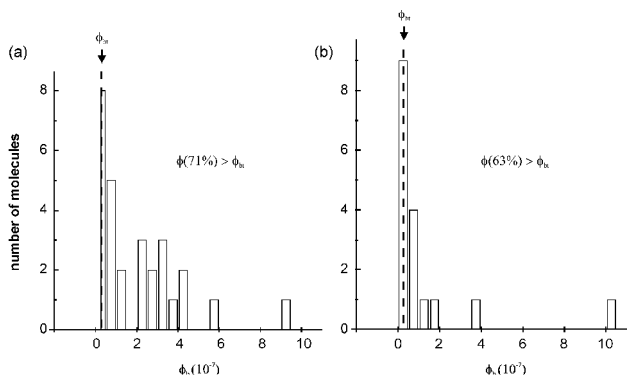


Figure 3. Histograms of the photo-destruction quantum efficiency  $\phi_b$  measured for photo-bleached molecules in the line scan experiment (22 ms per pixel): (a)  $I = 225 \text{ kW cm}^{-2}$ ; (b)  $I = 450 \text{ kW cm}^{-2}$ .

the excited molecule. The results are shown in figure 3(b). At this intensity, photo-bleaching was observed for all except one of the 18 investigated molecules. Now 63% of the molecules showed a photo-destruction quantum efficiency larger than the cutoff  $\phi_{bt}$  determined previously at half of the excitation intensity.

In the next experiment, molecules were placed in the excitation focus and continuously illuminated without scanning. The signal was sent to a multi-channel analyser (Stanford Research 430) with a 10 ms bin width. Fluorescence intensity traces (d–g) are presented in figure 4 for three different molecules (D, E, F). Molecule D showed a stable count rate of  $3.35 \times 10^5 \text{ counts s}^{-1}$  until photo-bleaching occurred after 18 s of photo-excitation (trace d). Trace e indicates an initially stable count rate for molecule E which suddenly started to fluctuate at  $t_1$  until bleaching occurred at  $t_2$  (see inset in figure 4). Similar fluctuations were observed for 15 out of 41 molecules in the line scan experiment but for only 2 out of 30 molecules in the continuous excitation experiment. Typical examples of fluctuations are shown in the traces of molecule C in figure 2 and in the traces of molecules E and F in figure 4. The fluctuations are characterized by some remarkable behaviour. The drops in intensity end at the background and the returns do not lead to the initial values in most cases. Smaller and larger count rates are recorded during the fluctuations if compared with the fluorescence intensity of the stationary periods. This effect was observed for all the mol-

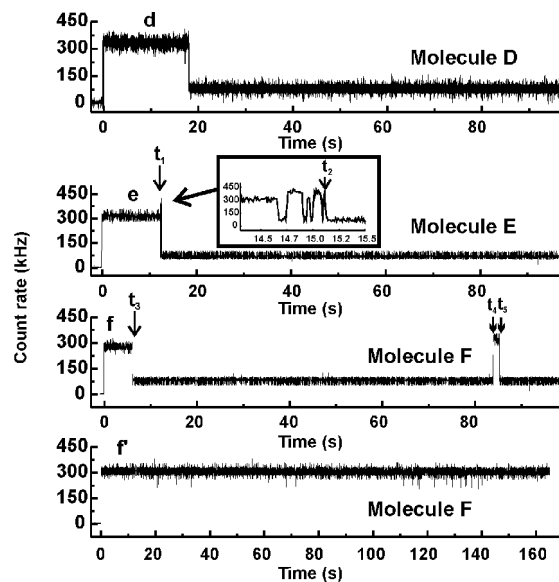


Figure 4. Fluorescence time traces for 3 different molecules excited continuously. A fluorescence on-off blinking is observed on a millisecond time scale for molecule E before photo-bleaching takes place at  $t_2$ .

ecules that underwent fluctuations. The fluorescence of molecule F ceased at  $t_3$ , recovered at  $t_4$  for a short period until bleaching occurred at  $t_5$  (trace f) and finally recovered again in a new acquisition (trace f') started a few seconds after the end of the measurement of trace f. Four consecutive traces were recorded for molecule F which showed exceptionally high photo-stability,  $\phi_b < 5 \times 10^{-10}$ . In these traces, the count rate decreased very slowly (probably due to mechanical drifts of the setup) from  $3 \times 10^5$  counts  $s^{-1}$  to  $2 \times 10^5$  counts  $s^{-1}$  during 11.5 min of photo-excitation.

Under continuous photo-excitation, 28 out of 30 molecules photo-bleached during the trace acquisition time of 128 s. The  $\phi_b$  measured at an excitation intensity of  $225 \text{ kW/cm}^2$  range from  $4.3 \times 10^{-9}$  to  $1.3 \times 10^{-7}$ . The corresponding ensemble-averaged photo-destruction quantum efficiency is estimated to be lower than  $\eta_{\phi_f}/\bar{N} = 1.2 \times 10^{-8}$  where  $\bar{N} = 5.7 \times 10^6$  is the average of  $N$  over the 28 photo-bleached molecules. The data are presented as a histogram in figure 5.

The survival probability of the molecules upon bleaching is assumed to decay exponentially at a constant bleaching rate  $k$  which depends on the excitation intensity. Under such conditions, the random numbers  $N$  of detected photons are exponentially distributed and, correspondingly, the  $\phi_b$  are expected to follow the distribution  $c \exp(-\phi_0/\phi_b)/\phi_b^2$  where  $c$  is a normalization factor. This distribution shows a maximum at  $\phi_0/2$ . With  $\phi_0$  taken as a fit parameter, the distribution was adjusted to the experimental data. The result is shown in figure 5. The parameter  $\phi_0$  is related to the decay rate  $k$

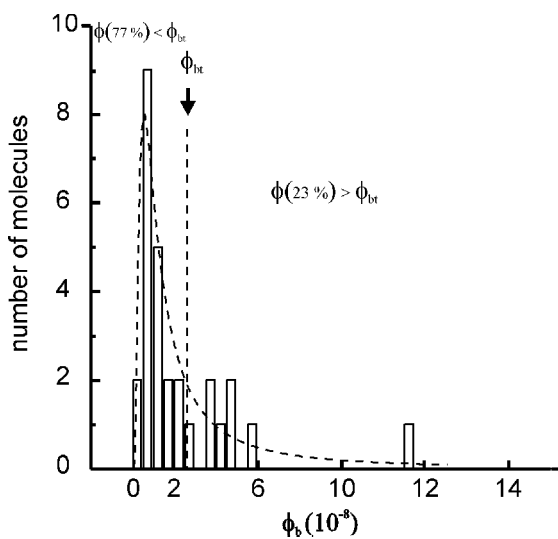


Figure 5. Histogram of the photo-destruction quantum efficiency  $\phi_b$  measured for photo-bleached individual molecules under continuous excitation ( $I = 225 \text{ kW cm}^{-2}$ ). The dotted line is a fit to the data (see text).

and  $\bar{N}$  by  $k/R = \phi_0/(\eta_{\phi_f}) = 1/\bar{N}$  where  $R$  is the fluorescence count rate. We found that  $\phi_0 \cong 1.2 \times 10^{-8}$  and  $k/R \cong 1.8 \times 10^{-7}$ .

Under continuous excitation, only 23% of the investigated molecules showed a  $\phi_b$  larger than the cutoff  $\phi_{bt} \cong 2.6 \times 10^{-8}$  defined above for the line scan experiment. Consequently, the photo-stability appears to be larger under continuous excitation.

We explain this result, as well as the fluorescence intensity fluctuations mentioned above, by assuming slowly diffusing quencher species in the sample. A quencher is considered to form, on contact with a molecule, a complex which emits no light upon photo-excitation. Because the quenchers diffuse presumably by hopping interstitially from one to another lattice site, they may take well defined positions relative to the terylene molecules. The random hopping of the quencher among these positions may thus lead to the characteristic fluctuations as observed in figure 4. We interpret the fluorescence enhancements by a shortening of the triplet lifetime through quenching of the triplet excitation. Indeed, the triplet state is supposed to be highly populated, as discussed below. Fluorescence intensity attenuation during fluctuations also is observed and considered to be an intermediate step towards complete bleaching. As a possible candidate for the quencher, we consider oxygen [20]. This assumption is supported by the results reported by Panzer *et al.* [21], who studied the influence of oxygen on the blinking of single molecules on a glass surface at room temperature. They observed that the fluorescence periods are shortened upon an increase in oxygen concentration while the dark periods of about 63 s are not affected. This result is explained by assuming that the dark periods depend mainly on the survival time of the oxygen–molecule complex.

The single molecule bleaching experiments described above differ in the ratio of on- and off-illumination periods. In the line scan experiment, a particular molecule is excited only for a short period compared with the scan duration, while in the experiment with fixed sample the molecule is continuously excited. Correspondingly, the total investigation time is much longer in the line scan experiment than for continuous illumination. The line scan experiments therefore favour the observation of the slow diffusion-controlled formation of quencher–molecule complexes. Because clearly these complexes have a survival time longer than the time required to perform one line scan ( $\cong 5$  s), it is very likely that a dark state due to complexing is detected. However, our interpretation holds only under the assumption that another photo-induced bleaching process dominates upon continuous excitation (see fit in figure 5). Indeed, while the molecules bleach on average after 1 min of continuous excitation, bleaching in the

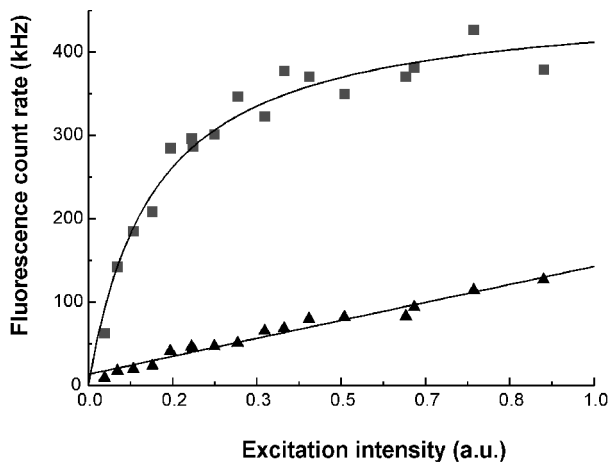


Figure 6. Single molecule saturation experiment:  $\square$ , fluorescence intensity above background as a function of the excitation intensity. The saturation intensity is  $450 \text{ kW cm}^{-2}$  and the saturation count rate is  $466 \text{ kHz}$ .  $\triangle$ , background intensity.

scanning experiment requires on average a time of 4 min. Therefore, we conclude that bleaching by diffusing quenchers dominates in the scanning experiment while photo-induced bleaching is the dominant process in the continuous excitation experiment. This explains also why a larger fraction of molecules exhibit fluorescence fluctuations during scanning ( $\cong 37\%$ ) than upon continuous excitation ( $\cong 8\%$ ).

The fluorescence intensity above background has been measured as a function of the excitation power for 17 molecules. All the molecular signals show power saturation behaviour that is fitted well by the saturation law of a two-level system. An example is plotted in figure 6 together with the average background count rate increasing linearly with the excitation intensity. The saturation intensity is about  $450 \text{ kW cm}^{-2}$ .

Two separated humps are clearly visible in the histogram of the saturation intensities in figure 7. The variation of apparent saturation intensity as a result of orientation of the molecular dipole with respect to the pumping laser polarization was reported previously in low temperature experiments for single terylene molecules in hexadecane [22]. The histogram of figure 7 indicates two different orientations of the dipole moment projections of the terylene molecules into the prominent  $(a, b)$  crystal plane of *p*-terphenyl [23, 24] which contains the laser field. The measured saturation count rates, plotted in the histogram of figure 8, are narrowly distributed with a mean value of  $430 \text{ kHz}$  and a maximum value of  $576.5 \text{ kHz}$ . These rates are comparable with those measured by Kummer *et al.* [14] at low temperatures. However, the detection effi-

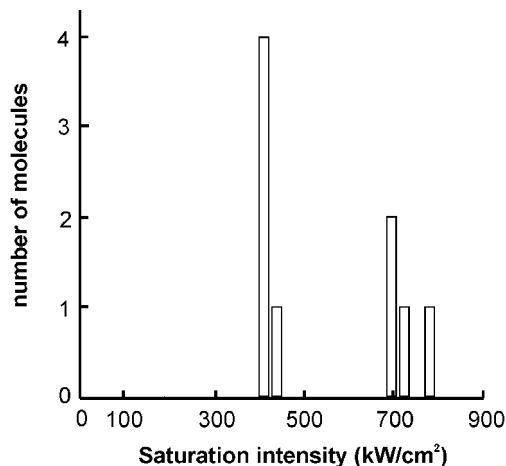


Figure 7. Histogram of the saturation intensities for 9 molecules with two distinct distributions around  $450 \text{ kW cm}^{-2}$  and  $700 \text{ kW cm}^{-2}$ .

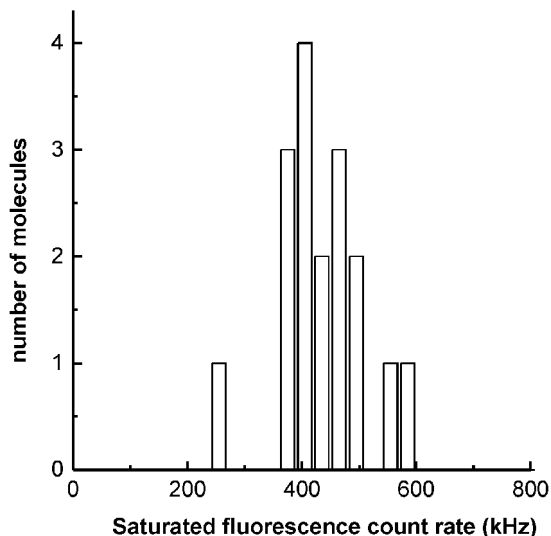


Figure 8. Histogram of saturated fluorescence count rates for 17 molecules with an average of  $430 \text{ kHz}$ .

ciency of our setup is roughly 10 times larger than the efficiency reported in [14]. We conclude therefore that the radiative rate contribution to the excited state lifetime  $\tau_1$  of terylene in *p*-terphenyl at room temperature is lower than at low temperature. We attribute this result to a larger number of competitive non-radiative decay channels available at room temperature. The large values of the saturation intensities also are consistent with a short  $\tau_1$  due to a rather strong non-radiative decay. However, high saturation intensities also may result if the molecular dipole moments have an unfavourable orientation relative to the laser polarization.

Such an effect was reported in [25] for the low temperature ordered phase of *p*-terphenyl.

In conclusion, the detection of single organic molecules in a molecular crystal at room temperature has been demonstrated. Terylene molecules rigidly immobilized in a *p*-terphenyl crystal show the largest photo-stability ever observed in a single molecule experiment at room temperature. This remarkable property makes the present system an ideal candidate for single molecule nano- and quantum optics experiments at room temperature.

The photo-stability of terylene was found to increase with increasing ratio of on- and off-illumination times. The largest photo-stability was found for continuous excitation. The reduced photo-stability in the line-scan experiment has been attributed to diffusion controlled formation of molecule-quencher complexes leading to no detectable fluorescence upon excitation. The fluorescence fluctuations observed after long stationary periods of stable emission have been assigned to quencher hopping in the vicinity of the terylene molecule.

Obviously, doped molecular crystals constitute a large class of promising new systems suitable for single molecule detection at room temperature.

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