Room-temperature single-molecule fluorescence imaging for terrylene in biphenyl single crystals

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Available online 6 November 2007

Abstract

The technique of room-temperature wide-field epifluorescence microscopy has been applied to visualize, on a single-molecule level, impurity-related dynamical processes in sublimation-grown flakes of biphenyl single crystals doped with terrylene molecules. For dilute samples, spatially resolved fluorescence of individual terrylene molecules can be observed using a standard microscope equipped with an EMCCD camera. The 532 nm excitation laser light induces irreversible photobleaching of single-molecule emitters; this process is inhibited in a nitrogen-enriched atmosphere, thus confirming the role of photochemical reactions between terrylene and oxygen. Although most of terrylene impurity molecules appear to stay fixed in the host crystal, some of them can be observed to move around over distances of tens of micrometers, probably diffusing along the defects of the crystalline structure.

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Keywords: Biphenyl single crystal; Terrylene; Single-molecule imaging; Room-temperature wide-field epifluorescence microscopy; Photobleaching; Diffusion

1. Introduction

Wide-field fluorescence microscopy has been used for single-molecule imaging (SMI) for already more than a decade. It was first applied for low-temperature studies of single impurity molecules (including terrylene (TR)) in different solids [1,2], and soon also for room-temperature (RT) SMI (mainly in biology-related [3,4], but also in studies on organic dye molecules in different host matrices [5–10]). TR molecules have so far been studied with RT SMI technique in a polystyrene film [10] and in thin p-terphenyl (PTP) single crystals (SC) [5,6,8], exhibiting in both cases irreversible photobleaching, but in the case of polymer host [10] also blinking behavior for part of the molecules. It is commonly assumed that the photobleaching of TR is caused by photochemical reaction with free oxygen (PRFO). Different populations of TR molecules have been observed in PTP SC, including the large stable population with extremely high resistance to photobleaching [5,6], and an unstable population of molecules traveling inside the crystal over distances of few micrometers [6].

Here we report on the first RT SMI study of TR in sublimation-grown biphenyl (BP) SC. This system has earlier been studied using the low-temperature techniques of single-molecule spectroscopy [11,12]. We investigate the photostability-related issues and report on observation of diffusing TR molecules.

2. Experimental

Few micrometer thick flakes of BP SC doped with TR have been grown using the co-sublimation technique, as in detail described elsewhere [11]; the concentration of TR molecules can be estimated as $\sim10^{-10}$ mol/mol.

The main difference of our experimental setup built for RT wide-field epifluorescence microscopy [7] from the setup recommended in the literature [3] is the use of an upverted microscope (Olympus MX14) with an air (not immersion) microscope objective having relatively low NA (Edmunds, 60 x magnification, NA = 0.9). This waste of efficiency is partially compensated by the use of Newton
EMCCD (CCD with electron multiplication on the chip) camera (Andor, matrix area $400 \times 1600$, pixel size $16\ \mu m$) as an imaging device. EMCCD technology allows to reduce the CCD readout noise in case of very low signals. The readout speed limits the time resolution to 0.1 s. To avoid an exposure during the CCD readout, an electromechanical shutter was installed in front of the camera. Commonly used exposure times were between 1 and 5 s; 200-frame movies have been recorded.

Frequency-doubled radiation ($\lambda = 532\mathrm{nm}$) of a CW Nd:YAG laser VA-532 (Viasho) was used for the excitation of TR molecules. The linearly polarized laser beam was expanded by a telescope and reflected by a dichroic mirror XF2012 (Omega Filters) into the microscope objective. The telescope lenses were adjusted to focus the excitation beam onto the back-focal plane of the microscope objective, creating an illuminated area of about $40\ \mu m$ in diameter on the sample. Fluorescence from the sample was collected by the microscope objective and transmitted through the dichroic mirror. The backscattered laser light was blocked by a holographic Super Notch filter HSPF-532.0-1.0 (Kaiser) and an orange long-pass glass filter. After passing through the filters, the fluorescence was imaged onto the CCD camera by a 200 mm achromatic lens system. Eventually, 1 pixel of the camera image corresponds to $105\ \mu m$ on the sample.

Samples were placed on an ordinary 0.3 mm microscope cover glass (Knittel), which could contribute to the background signal level. Under excitation intensity of $1.1\ kW/cm^2$, an average registered background level was about $1650\ \mathrm{counts/s\ pixel}$, and the fluorescence level of intense molecules was about $600\ \mathrm{counts/s\ pixel}$ over the background (to find this number, we summarized the signals registered by 49 pixels located in an 8 pixel diameter round area around the center of the corresponding bright spot and normalized the result to one pixel). A special computer program was created to analyze the fluorescence kinetics of many individual molecules registered in a single movie.

3. Results and discussion

In Fig. 1, typical observed fluorescence dynamics of TR single molecules in BP SC under constant laser irradiation is presented. Similarly to the case of PTP SC [5,6], all the molecules are experiencing single step photobleaching with no blinking behavior. But differently from that case, no different populations of single emitters with significantly different photobleaching probabilities could be detected with 2 s time resolution. As can be seen from Fig. 1b, the population decay is nearly single-exponential, and virtually no single emitters survive after several minutes of laser excitation. On the other hand, in a nitrogen-enriched atmosphere (under nitrogen gas flush), the photobleaching is significantly inhibited, being over four times slower than under the normal conditions. This result is consistent with an assumption that the photobleaching of TR is caused by PRFO.

Fluorescence saturational behavior of a sample TR impurity has been analyzed and the conclusion made that with our excitation intensities we are well below the saturation conditions (see Fig. 2 and the caption).

In Fig. 3a, the typical recorded SMI image is presented (see the caption for details). This image is just a frame in a 400 s movie, taken with 2 s time resolution. A 96 s sub-movie starting with this frame contains a moving emitter (with initial position indicated by an arrow), which travels over the distance of at least $17.5\ \mu m$ and then disappears (photobleaches similarly to many of its virtually fixed neighbor TR molecules). Fluorescence intensity fluctuations in a smaller zoomed-in crystal area during this...
sub-movie are highlighted in Fig. 3b (see the caption for details). The traveling molecule moved with average speed of 0.181 m/s, but the highest speed we registered with our time resolution was over 0.66 m/s.

Other moving emitters could be observed in several movies recorded, but they could be traced only for relatively short times, probably due to their poor photostability.

4. Conclusions

We have shown that wide-field RT SMI experiments can be performed with a setup based on a standard microscope equipped with an EMCCD camera; the first SMI experiments for TR molecules doped to BP SC are reported.

All TR impurities we observed so far at RT suffered irreversible photobleaching under the 532 nm laser excitation, with no blinking behavior. Only one population of TR emitters in respect to their photobleaching quantum efficiency could be revealed with 2 s time resolution. Still, photobleaching is strongly inhibited in a nitrogen-enriched atmosphere, which is consistent with the commonly assumed photobleaching mechanism, involving PRFO. Further experiments would be necessary to investigate the photobleaching mechanism in detail.

We report observation of diffusing TR molecules, able to travel in BP SC over distances of more than 15 µm. Observed diffusion tracks are significantly longer than those earlier observed in much thinner spin-coating-prepared PTP SC [6]. Dedicated experimental and theoretical studies will be necessary to investigate the physical mechanisms involved.

Acknowledgments

Authors are grateful to Dr. I. Sildos for constant interest and infrastructural support. Support from Estonian Science Foundation Grants no. 7141 and 6543 is also acknowledged.
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