

Single-molecule Imaging with an Inexpensive UV-LED Light Source

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The powerful tool of single-molecule detection and spectroscopy has so far been confined mainly to the visible spectral region. Here, we report the use of a low-cost high-power UV light emitting diode (LED) emitting at 365 nm as a light source for single-molecule detection in epi-fluorescence microscopy. The LED provides an affordable alternative to UV laser systems. The performance of the light source is demonstrated by recording fluorescence images and time-dependent fluorescence traces of single molecules of a laser dye *p*-bis(*o*-methylstyryl)benzene (bis-MSB) and of a conjugated polymer polyfluorene.

In recent years, single-molecule optical detection and spectroscopy have developed into standard techniques for the study of structural and dynamic properties of materials and biological systems at nanometer scales.¹ The methods are based on spatial isolation of immobilized dye molecules in extremely dilute samples. Individual molecules are detected via their fluorescence signals using scanning confocal or wide-field optical microscopies. So far, single-molecule experiments have been almost exclusively confined to the visible and near-infrared regions of the electromagnetic spectrum. Reports on UV excitation on single-molecule level include use of the second harmonic of a Ti:Sapphire laser at 350 nm to study one- and two-photon dynamics of Coumarin-120² and Rhodamine 6G³ dyes, and at 360 nm to investigate H-258 DNA complexes⁴ and multichromophoric dendrimers.⁵ Recently, deep-UV diode-pumped picosecond laser at 266 nm has also been used for imaging of *p*-quaterphenyl dye⁶ and for label-free detection of single protein molecules.⁷ Apart from the common difficulties associated with UV excitation, such as increased background due to impurity fluorescence and increased quantum efficiency of photobleaching, the lack of research reports on UV-excited single-molecule detection can be ascribed to the lack and high cost of suitable light sources. However, recent progress in the development of high-power UV light emitting diodes (LED) on the one hand, and ultra-sensitive electron-multiplication CCD camera detectors (EM CCD) on the other may change the situation. Low-cost LEDs with powers of more than 100 mW in the near UV region are now widely available, and the decreased read-out noise of the EM CCD detectors has lowered the necessary energy density for single-molecule excitation. The routine accessibility of the UV region in single-molecule studies might open new areas of materials and biological systems for study on nanometer scales. In this letter, we report on the construction of an inexpensive light source based on a high-power LED emitting at 365 nm and demonstrate its performance by imaging single molecules of a laser dye, *p*-bis(*o*-methylstyryl)benzene (bis-MSB) and of a conjugated polymer polyfluorene.

The experimental setup is based on an inverted optical microscope (Nikon Diaphot 300). The scheme of the setup is shown in Figure 1. High-power chip-type UV LED (Nichia Corp.

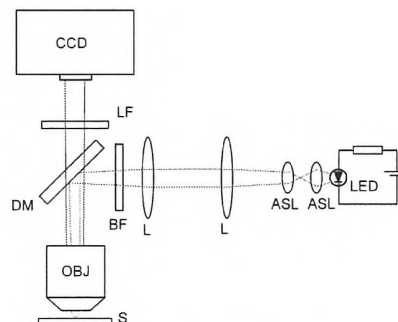


Figure 1. Scheme of the experimental setup. LED: high-power UV LED; ASL: aspheric lens; L: convex lens; BF: band-pass filter; DM: dichroic mirror; OBJ: microscope objective lens; S: sample; LF: long-pass filter; CCD: EM CCD camera.

NCCU 033) has a typical optical output power of 100 mW at 365 nm and spectral width of 10 nm. The LED is encased using an aluminum block and powered with a 5 V dc power adapter. The emitted light is collimated with two aspheric lenses ($f = 3.1$ mm and $f = 11$ mm, Thorlabs) and a convex lens ($f = 100$ mm) into slightly divergent beam. The beam is further focused to the back focal plane of the used microscope objective with a convex lens ($f = 170$ mm). Autofluorescence of these optical elements is cutoff with a band-pass filter at 360 nm (U-360, Edmund Optics). The beam is directed into the microscope in an epi-fluorescence mode using a dichroic mirror (Olympus, 420 nm). For the single-molecule imaging experiments three objective lenses were tested: 100 $\times$ air (Olympus, UPlanFL100 $\times$, N.A. = 0.95), 100 $\times$ oil-immersion (Olympus, UPlanFLN100 $\times$ O2, N.A. = 1.3) and 40 $\times$ air for UV excitation (Olympus, UApo40 $\times$ 3/340, NA = 0.9). The best results were obtained using the oil-immersion lens and nonfluorescent immersion oil (Cargille Lab., DF). The data presented in the rest of the paper were recorded using this lens. Fluorescence from the sample passes a 400 nm long-pass cutoff filter (LUX400, Asahi Spectra) and is detected with an EM CCD camera (iXon+, Andor).

The laser dye, bis-MSB (absorption maximum 350 nm, fluorescence maximum 418 nm), was purchased from Kanto Chemical. The polymeric dye, poly(9,9-di-*n*-octylfluorenyl-2,7-diyl) (absorption maximum 365 nm, fluorescence maximum 440 nm), was supplied by Aldrich. The dyes were dissolved in 1.5 wt % toluene solution of Zeonex 480R (Zeon) to achieve a final concentration on the order of 10^{-11} M. Thin polymer films (thickness ≈ 100 nm) were prepared by spin-coating small amount of the dye/polymer solution on quartz cover slips (Electron Microscopy Sciences) at 3000 rpm for 20 s. The films were vacuum-dried at 60 $^{\circ}$ C for several hours. All experiments were done at ambient conditions.

The first system for demonstrating the light source perform-

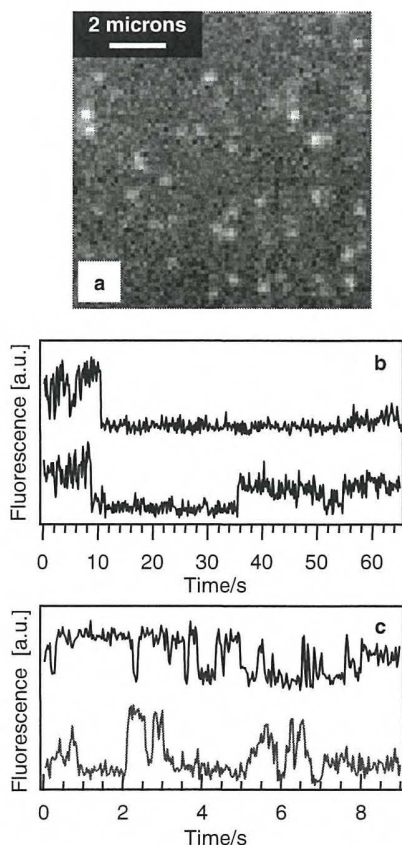


Figure 2. a: Microscopic image of single molecules of bis-MSB in a Zeonex matrix. Integration time 600 ms, excitation intensity 16 W cm^{-2} . b: Time-dependent fluorescent traces of two molecules of bis-MSB. Binning time 150 ms, background subtracted. c: Time-dependent fluorescent trace of a single molecule of polyfluorene in a Zeonex matrix. Binning time 50 ms, background subtracted.

ance, the molecule bis-MSB, was chosen for its high molar extinction coefficient at 350 nm and for being known as an efficient laser dye.⁸ A typical single-molecule fluorescence image obtained with 600 ms integration time and maximum gain of the EM CCD camera is shown in Figure 2a. The excitation intensity (measured above the objective lens and normalized by the irradiation area at the sample) was 16 W cm^{-2} , which is in the lower range of intensities used typically for single-molecule detection. Although the background is relatively high (the signal-to-background S/B ratio is approximately 1:3), the image shows good signal-to-noise (S/N) ratio. The S/N ratio was determined from the signal after background subtraction. The S/B ratio of 1:3 is the best value of several substrate/matrix combinations tried. With glass cover slips and/or poly(methyl methacrylate) matrix we were not able to detect any single-molecule images due to the high background. The residual background might be reduced further by the use of non-oil-immersion objective lenses (such as water or glycerol type immersion lenses) or quartz objectives.

The bis-MSB single molecules exhibit the usual single-molecule time-dependent attributes of blinking and single-step

photobleaching. Figure 2b shows typical time-dependent fluorescence intensity trajectories of two molecules. The data were taken with binning (the CCD integration time) of 150 ms. The top trace shows a molecule photobleaching at approximately 10 s, while the bottom trace represents blinking between several intensity levels. The S/N ratio at this binning is more than four. As also seen from the figure, most molecules continue emitting for relatively long times of seconds to tens of seconds before photobleaching. This fact which can partly be attributed to the low excitation intensity used, proves that a careful choice of the system enables prolonged studies of single fluorescent molecules even with near-UV excitation.

The second system studied was the conjugated polymer polyfluorene. While single-molecule studies of conjugated polymers continue to bring new insights into the polymer conformations and photophysics,^{9,10} polyfluorenes have so far escaped attention despite their important role as charge-transport and blue-emitting materials.¹¹ Here, the Figure 2c shows time-dependent fluorescence traces of two polyfluorene single molecules. Good S/N ratio (the traces in Figure 2c have $S/N \approx 7$) can be obtained with binning time as short as 50 ms. As in the case of other conjugated polymers such as MEH-PPV,⁸ single polyfluorene chains exhibit on-off blinking behavior between two or a few fluorescence intensity levels, pointing to the presence of fast energy transfer and localization of the energy on a few emitting centers.

In summary, we have demonstrated that single-molecule detection with good signal-to-noise ratio in wide-field microscope can be performed using a low-cost home-made UV LED light source. Our attempts to use the light source in confocal microscope have failed because of the difficulty of collimating the diode light into a narrow parallel beam of sufficient intensity. The cost-performance of the source (the total cost was approximately 500 USD) is more than two orders better than laser sources operating at this wavelength. We believe that this report will open the way for other laboratories to extend the single-molecule research into the near UV spectral region.

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