Development of new microscope unit for single molecule spectroscopy under various ambient conditions

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Abstract. This paper introduces techniques we previously developed for single molecule spectroscopy and continues on to describe our studies on dipole orientation imaging of single molecules under various ambient conditions. In these studies, we successfully obtained defocused images of single perylene diimide (PDI) molecules under air, high-vacuum, and pure N₂ gas conditions by utilizing the advantages of our new microscope unit. The studies are positioned as one of the important applications of our microscope unit for single molecule spectroscopy. We expect a wide range of applications for this unit for various microscope measurements for many types of materials.

1. Introduction

Single molecule spectroscopy, along with use of the time-correlated single photon counting (TCSPC) system, has been widely applied in studying the emission characteristics of individual organic fluorescent molecules, colloidal semiconductor nanocrystals, and biomolecules labeled with fluorescent moieties [1,2].

We have developed new techniques for efficiently performing single molecule spectroscopy. First, we demonstrated the usefulness of the TCSPC system with modified photomultiplier tubes as photon detectors, which have high quantum efficiency, a relatively large active area, relatively high response speed, and extremely small dark counts [3,4]. Our system has sufficiently high sensitivity for performing single molecule spectroscopy by utilizing the above advantages. We then reported the fabrication of a two-dimensional photonic crystal (PC) slab using tantalum pentoxide (Ta₂O₅) that has low background emission [5]. Enhancement of fluorescence from single molecules of N,N’-bis(2,6-dimethyl-phenyl)perylene-3,4,9,10-tetracarboxylic diimde (PDI) on a Ta₂O₅-PC slab with low background emission was observed due to efficient coupling of excitation and fluorescence light to PCs’ modes [6]. This was the first demonstration of enhancement of fluorescence from single fluorescence dyes via the effect of two-dimensional PCs. Then we developed a new microscopy unit that has an immersion objective with a high numerical aperture (NA) (1.3-1.4) and an ionic liquid as a non-volatile refractive index matching medium. The unit enabled us to perform single molecule spectroscopy in a high vacuum (10⁻⁵-10⁻⁶ Torr) and ambient gas with an appropriate pressure [7]. The microscopy unit was applied to single molecule spectroscopy for single colloidal quantum dots (CdSe/ZnS core shell nanocrystal), and the differences of the optical characteristics between in air, high vacuum, and pure N₂ gas were elucidated [8]. Moreover, we performed studies toward
optimization of the microscope unit [9]. Thus, we have developed three techniques toward a wide range of applications of single molecule spectroscopy as well as an efficient form of this spectroscopy. In this article, we focus on the studies on dipole orientation imaging of single PDI molecules under air, high vacuum, and pure N₂ gas conditions. These studies are positioned as another important application of our microscope unit for single molecule spectroscopy. We would like to emphasize a wide range of applications of this unit for various kinds of microscope measurements for numerous types of materials.

2. Experimental procedures
Figure 1 shows the schematic of our laser system and new microscope unit. The main differences compared with our previous studies are the excitation light source and detection system. In the present study, we used a continuous wave (cw) laser with a wavelength of 488 nm as an excitation light source and a charge coupled device (CCD) camera as a detector, while in the previous studies, we used a laser with a wavelength of 446 nm or 488 nm, pulse width of 2 ps, and repetition frequency of 8 MHz as an excitation source and a modified PMT (H7422P-40-MOD, Hamamatsu) as a detector. We thus performed epifluorescence microscopy with wide-field illumination for dipole orientation imaging rather than the confocal microscopy in previous studies.

![Figure 1. Schematic of laser system and new microscope unit](image)

Our microscope unit is essentially the same as in previous studies [8,9]. It consists of a turbo molecular pump (TMP), gas purge line, small vacuum chamber with a synthesized quartz window, sample substrate, XY- and Z-translation stages, immersion objective with an NA of 1.3 and magnification of 100×, and an ionic liquid. As previously shown, the inside of our microscope unit also offers ambient gas conditions at an appropriate pressure, as well as high-vacuum conditions. The phosphonium-based ionic liquid (IL-AP3-3, Koei) as shown in figure 2(b) with a refractive index of 1.5 was used as non-volatile refractive index matching medium [9]. This ionic liquid has low background fluorescence comparable to that of non-fluorescence (NF) immersion Oil (Nikon) and a good refractive index matching with a general glass coverslip [9].

We prepared a toluene solution containing PDI as shown in figure 2(a) and 0.5 wt% poly(methylmethacrylate) (PMMA). The PDI/PMMA/toluene solution was spin cast onto a pre-cleaned glass coverslip substrate at 3000 rpm. This sample substrate was used in our experiments.

We performed epifluorescence microscopy of the sample with wide-field illumination by using a 488 nm cw laser. The fluorescence from the sample was collected by the same microscope objective and detected by an electron multiplying (EM) CCD camera (iXon3, Andor), after passing through a
dichroic mirror, relay lens, and edge filter to eliminate the excitation light. The dipole orientation images were obtained by defocusing about 1 μm displacement of the immersion objective toward the sample substrate.

Figure 2. Chemical structures of (a) PDI and (b) ionic liquid, IL-AP3-3

3. Results and discussion

First, we confirmed the usefulness of the immersion objective with the ionic liquid, IL-AP3-3. The microscope unit shown in figure 1 was used without vacuum evacuation, that is, in air. Figures 3(a) and 3(b) show the detected images at the focal plane and by defocusing, respectively, obtained by using the ionic liquid and immersion objective. The detected images were essentially the same as those by using the immersion objective with Nikon NF immersion oil. These results indicate that the ionic liquid works as a good refractive index matching medium with low background fluorescence. We also tried to obtain defocused images by using a general high NA (0.95) microscope objective that is used without a refractive index matching medium. However, we failed to obtain defocused images similar to figure 3(b). Thus, the use of an immersion objective and refractive index matching medium is necessary for obtaining defocused images, as found a number of studies [10-12].

Figure 3. Detected images (a) at focal plane and (b) by defocusing, obtained by using the ionic liquid and the immersion objective in air. Bright spots in (a) correspond to single PDI molecules.

Figure 4(a) shows a typical defocused image obtained by using the microscope unit under a high vacuum condition; thus, we successfully obtained the defocused image. Figures 4(b) and 4(c) are selected magnified images. In Fig. 4(b) are found concentric rings in which the center is dark, and the corresponding dipole orientation has vertical orientation with respect to the surface plane. Figure 4(c) is found a typical dipole radiation pattern and the corresponding dipole orientation has parallel orientation with respect to the surface plane. Böhmer and Enderlein [10] investigated the relationship between the defocused images and dipole orientation. During the measurements in high vacuum, we observed a relative temporal stability of defocused images that would originate from inhibition of the
photobleaching in the high vacuum. Figure 4(d) shows a typical defocused image obtained by using the microscope unit in pure N₂ gas with an appropriate pressure (0.12 MPa); thus, we successfully obtained the defocused image in ambient gas conditions. We for the first time succeeded in obtaining defocused images of single PDI molecules under air, high vacuum, and pure N₂ gas conditions, by utilizing the advantages of our microscope unit.

![Figure 4](image)

**Figure 4.** (a) Typical defocused image obtained by using the microscope unit under a high vacuum condition. (b) and (c) are selected magnified images. (d) Typical defocused image obtained by using the microscope unit in pure N₂ gas with an appropriate pressure (0.12 MPa)

### 4. Conclusions
We introduced the techniques that we previously developed for single molecule spectroscopy and then described the recent studies on dipole orientation imaging under various ambient conditions. In these studies, we successfully obtained defocused images of single PDI molecules under air, high-vacuum, and pure N₂ gas conditions by utilizing the advantages of our microscope unit. The application of our microscope unit is not restricted to single molecule fluorescence spectroscopy under various ambient conditions. It is expected to have a wide range of applications, such as surface plasmon, and Raman scattering, and nonlinear optical microscopy, which require high resolution and high brightness under various ambient conditions. We expect many applications for the unit in the fields of analysis of organic and inorganic thin films, and in nano-technology and bio-technology.

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