

Imaging Single Fluorescent Molecules at the Interface of an Optical Fiber Probe by Evanescent Wave Excitation

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We have developed a new fluorescent method for single-molecule detection (SMD) and imaging using an optical fiber probe. The fluorophores were excited by the evanescent wave field produced on the core surface of the optical fiber. This was achieved by exposing a section of the core of the optical fiber probe to the fluorophore solution. Both cylindrical and square optical fiber probes were used for SMD. The fluorescent signals were detected by an intensified charge-coupled device. Single rhodamine 6G molecules have been detected. The number of rhodamine 6G molecules imaged by the optical fiber probe showed an excellent linear relationship with the concentrations of the fluorophores. The SMD scheme was also applied to the imaging of biomolecules, such as molecular beacon DNA molecules, labeled with tetramethylrhodamine. Our results have shown that using an optical fiber is an easy yet effective approach to SMD. It represents a simpler fluorescent method for the detection of single-molecules in solution and at an interface.

There have been recent advances in applying fluorescence techniques for the observation of species at the level of individual molecules.^{1–12} Single-molecule detection (SMD) represents the ultimate goal of ultrasensitive chemical analysis and potentially leads to the study of molecular mechanisms of biomolecular functions, reactions, and their diagnostic applications in medicine.

As many features important for biological systems can only be realized in an aqueous environment, single-molecule detection in solution is of increasing interest. Methods have been developed for detecting dye molecules and dye-labeled biomolecules in flowing streams,^{1,9,12,13} levitated microdrops,¹⁴ and static solutions^{4,5,7} and during capillary electrophoresis.^{15,16}

The challenge for SMD in solution is to extract signal from high background due to light scattering and fluorescent impurities.¹ The signal-to-noise ratio can be increased in a number of ways. One approach is to improve the detection capability of SMD systems by using a mode-locked pulse laser,^{12,13} an avalanche photodiode (APD),^{4,9,12,13} an intensively charge-coupled device (ICCD),^{5,7} or a refinement of the arrangement of the optics.⁵ Another approach is to work with a very small probe volume (less than 1 pL) to reduce the background signal. The observation or excitation volume can be confined optically by the use of a confocal microscope^{4,15} or evanescent wave excitation.^{5–7}

An evanescent wave field is produced by total internal reflection at an interface. Its intensity decays exponentially from the interface, usually resulting in a penetration depth of less than 350 nm depending on the wavelength of light being used for excitation.² The scheme of exciting single fluorophores in an evanescent field has been employed by a few research groups.^{5–7} Funatsu et al.⁵ used refined total internal reflection fluorescence microscopy to visualize single fluorophores in solution and observed ATP turnover reactions. Tokunaga et al.⁵ recently modified epifluorescence microscopy to an objective type of total internal reflection fluorescence microscopy for SMD. Dickson et al.⁶ reported evanescent wave excitation at the boundary of a cover slip and a polyacrylamide gel for the detection of fluorophores diffusing in and out of the gel. With an ICCD, Xu and Yeung⁷ measured the diffusion and photodecomposition of single-molecules in solution and studied the electrostatic trapping of protein molecules at a liquid–solid interface. In all the above methods, an optical prism based system has been used as the waveguide for the generation of the evanescent field.

We have developed a simple method for imaging single fluorescent molecules using an evanescent wave produced on the

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core surface of an optic fiber. The evanescent wave is a fundamental characteristic of an optical fiber waveguide. Fiber-optic evanescent wave biosensors have been used for real-time detection of a variety of biomolecules and the study of their reactions, such as antibody–antigen binding and DNA hybridization.^{17,18} They are becoming a popular analytical method in biomedical and biochemical applications.^{19,20} This work is intended to use fiber-optic evanescent wave excitation for easy and effective single-molecule detection. We expect that the optical fiber based SMD technique will benefit from the rich database of fiber-optic sensor technology.^{19–21} In this work, an optical fiber will be used to probe into a fluorophore solution or at a solid surface. The fluorophores in the proximity of the optical fiber surface are excited by the evanescent wave. The fluorescent signals generated by the fluorophores are detected by an ICCD-based microscope system. It is worth noting that the collection of fluorescence in our SMD system is different from that used for the conventional evanescent fiber-optic sensors, in which the emission signals are coupled back to the fiber for detection. In addition to the conventional cylindrical fused-silica fiber, a new type of plastic square fiber is also used for SMD. The introduction of an optical fiber probe for SMD simplifies the optical arrangement and may expedite the practical applications of SMD techniques.

EXPERIMENTAL SECTION

Reagents. Rhodamine 6G (R6G, Acro, NJ) and R6G-dUTP (PE Applied Biosystems, Foster City, CA) were dissolved in deionized water from a Millipore Milli-Q system. A fluorescein (Eastman Kodak, Rochester, NY) solution was prepared in 20 mM phosphate buffer (pH 8.0). The molecular beacon,²² which has a sequence of 5'-(TMR) CCT AGC TCT AAA TCG CTA TGG TCG CGC TAG G(DABCYL)-3', was developed in our laboratory.²³ It was dissolved in Tris/HCl buffer (20 mM Tris/HCl, 50 mM KCl, and 5 mM MgCl₂, pH = 8.0). The water and the buffers were pretreated by photobleaching with 2 h intensive illumination of an argon ion laser beam and filtering through a sterile 0.2 μm cellulose acetate membrane (Corning Glass Works, Corning, NY).

Optical Fiber Probe Preparation. Cylindrical fused-silica optical fibers with 365 μm or 1 mm core diameters were purchased from Newport Corp (Irvine, CA). The exposure of a section of their core surfaces was achieved by chemical etching. First, about 5 cm of the jacket at one end of a fiber was removed. Then the fiber was perpendicularly dipped into a 4 cm deep 49% hydrofluoric acid solution (Fisher Scientific, Pittsburgh, PA) for 30 min. The HF solution was covered by isoctane solvent (Fisher Scientific) as a protection layer during the etching process. The etched fiber probe was sufficiently washed with water before applications.

A plastic square fiber with a 1 mm width core was obtained from Nanooptics Inc. (Gainesville, FL) and was cut to about 30

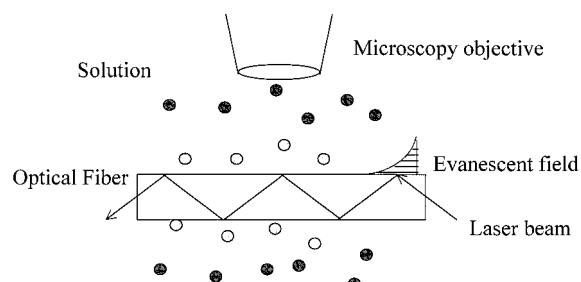


Figure 1. Instrumental setup for single-molecule detection and imaging in solution using an optical fiber probe.

cm in length. At one end of the fiber, the cladding was mechanically peeled off to expose a 4 cm long core area. After cleaning the core by ethanol, the fiber was put into a 70 °C oven for pretreatment.

Instrumentation. Figure 1 schematically depicts the setup for the detection of single-molecules in solution. The system mainly consists of a microscope, an ICCD, an argon ion laser, and an optical fiber probe sitting on the microscope stage. An upright microscope (Olympus, model BX40F4) is used for optical measurements. The microscope was equipped with a 100× (Olympus, LM Plan F1, 0.8 NA) and a 40× (Olympus, UplanFI, 0.75 NA) objective. All images for rhodamine dye molecules were taken by the 100× objective, while the images for fluorescein dye molecules were taken by the 40× objective. An ICCD (Princeton, EEV 512 × 1024 FT) was mounted on the top entrance port of the microscope. Excitation laser beams, which were 514 nm for rhodamine dyes and 488 nm for fluorescein, were produced from an Innova 307 Ar⁺ laser (Coherent Laser, Santa Clara, CA). The laser power was 50–100 mW. The laser beam was directed to one end of the optical fiber probe through an optical fiber coupler (Newport Corp., Irvine, CA). The other end of the fiber was inserted into a homemade plastic channel (3 × 2 × 0.2 cm). The channel was placed on the stage of the microscope and filled with a sample solution. The evanescent field was generated on the surface of the exposed core area of the fiber and was used to excite the fluorophores in the solution. Fluorescent signals thus produced were collected by the objective and then directed to the ICCD. To specifically select the fluorescent signal, a 530 nm long-pass filter and a 550 nm interference filter were put in front of the ICCD camera for the detection of R6G. The filters were changed to a 500 nm long-pass filter and a 515 nm interference filter for fluorescein monitoring and to two optical filters (550 and 570 nm) for the molecular beacon. The ICCD was controlled by a PC computer with WinView software (Princeton Instruments, Trenton, NJ). Images were taken using a subframe of 50 × 50 pixels or 50 × 100 pixels. The exposure times used in all the experiments for the collection of an image of single-molecules were 100 ms.

RESULTS AND DISCUSSION

Evanescent Wave Excitation for Single-Molecule Imaging.

The evanescent field generated on a cylindrical optical fiber surface has been well characterized and studied.²⁴ For a multi-mode cylindrical fiber, as its diameter is much greater than the excitation wavelength, the individual reflections of the guided excitation rays from the core-cladding interface can be considered as reflections from a planar interface. The fiber thus can be

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characterized by adding the contributions from all the propagated individual rays. It is expected that the evanescent field generated on the fiber core surface is similar to that by a prism, but much more uniform, especially for a larger area. By utilization of the evanescent field, the multimode cylindrical fibers have been widely used as evanescent wave biosensors for a variety of applications.^{17–20} The evanescent field on the surface of an optical fiber probe is uniform,²⁴ with both the space and the imaging integration time used in this work (100 ms), and sufficiently strong for single-molecule detection.

The evanescent field has a very small penetration depth in the vertical direction and thus only excites the fluorophores exclusively in the close proximity of the probe surface. This greatly reduces the background signal from the bulk solution, minimizes the detection volume, and enables investigation of surface phenomena. The observation volume of the ICCD setup is determined by a few factors, including the physical size of each pixel, the magnification of the microscope, the evanescent field depth, and the way the intensifier is coupled. The pixel size of the ICCD camera is 23 μm determined by calibration using a standard microscopic grid. As the depth of the evanescent field in our experiment is about 250 nm, we estimated that one pixel of the ICCD only detects a volume of 3×10^{-17} L when a 100 \times objective is used. This is a sufficiently small volume to isolate individual molecules even with a fluorophore concentration in the 10^{-7} M range. With a 40 \times objective, the fluorophore concentration for SMD is about 10^{-8} M. When more diluted fluorophore concentrations, such as 10^{-8} M for a 100 \times objective and 10^{-9} M for a 40 \times objective, are used, the probability of detecting two molecules at the same pixel is negligibly low. This is one of the most important considerations in realizing SMD.

Cylindrical Fused-Silica Fibers for Single-Molecule Detection. Conventional cylindrical fibers have been used for single R6G and fluorescein molecule detection and imaging. It is usually not easy to focus on the surface of a cylindrical optical fiber probe, especially when the diameter of the fiber is small. However, we were still able to focus on the fiber surface with subframe imaging without any difficulties. This was especially true when a long working distance microscope objective of 100 \times was used. When the core diameter of the fiber was increased to 1 mm, the focusing of the optical fiber top surface became conventional.

Figure 2 shows the imaging of both pure water and individual R6G molecules using a cylindrical fiber with a 100 \times objective. The sample channel was first filled with water, and a group of images (8–10) were taken for the blank. A threshold was set according to the photoelectron count distribution obtained from these images. The threshold was chosen at a value of 3 times the standard deviation (3σ) above the average of photoelectron counts for these images. Statistically, more than 99.7% of the pixels have lower intensity counts than the threshold value if there are no dye molecules in the solution.²⁵ There were only a few bright spots displayed in the image obtained from water, as shown in Figure 2A. In this specific experiment, the threshold value was determined to be 27. If there were many bright spots in the blank

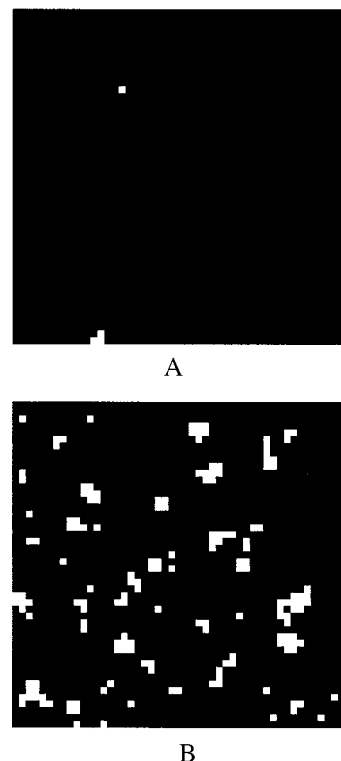


Figure 2. Fluorescent image (50 \times 50 pixel subframe image) of single R6G molecules in water: A, purified water; B, 1.7×10^{-8} M R6G solution. A 365 μm core cylindrical fiber was used.

solution images, it indicated that the blank solution would need to be purified further. After the experiment with water, the solution in the channel was replaced by a 1.7×10^{-8} M R6G solution. By application of the same threshold, many bright spots were shown in the images for the R6G solution under the same experimental conditions (Figure 2B). These bright spots indeed represented R6G molecules in the solution. Most of the bright spots occupy more pixels than the spots observed in water. This is due to the lateral diffusion of R6G molecules within the evanescent field depth, which has been observed previously by Xu and Yeung when a prism setup was used for SMD.⁷ Taking account of the R6G concentration and the detection volume of each pixel, we see that each bright spot in Figure 2B corresponds to the fluorescent signal from a single molecule.

There are three observations in Figure 2B to support that single R6G molecules were indeed imaged. First, statistically, the detection volume and the R6G concentration only enabled at most one molecule at each pixel, with the vast majority of the pixels without any molecules. Second, the number of single-molecules imaged in our experiments were in close agreement with the R6G concentration used, taking account of the established factors in fluorescence detection efficiency.^{1–3} Third, there is an excellent linear relationship between R6G concentration and the number of single-molecules observed in the images. As shown in Figure 3, the number of the bright pixels was proportional to R6G concentrations. Usually, the small detection volume and the fluorophore concentration are used to claim SMD in solution,^{5–7,9,10} which is mainly a statistical argument with a high confidence of the conclusion. Here, our SMD claim is further supported by the linear relationship between the number of single-molecule events detected and the concentration of the molecules. We detected

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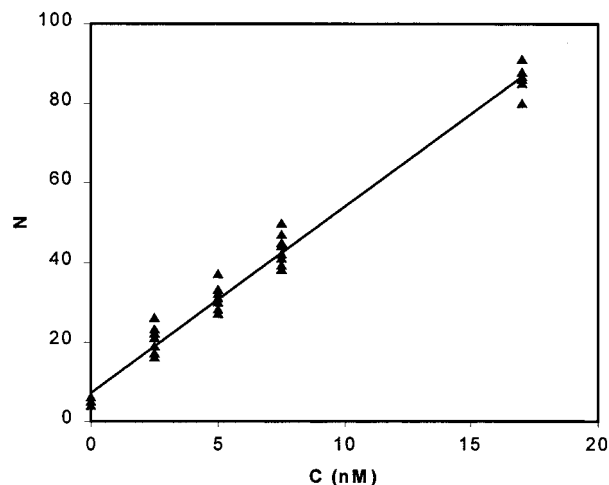


Figure 3. Number of individual R6G molecules imaged in solution with different R6G concentrations. Results were obtained from 50×100 pixel subframe images. The images were taken under the same experimental conditions as those for Figure 2.

single R6G molecules from 2.5×10^{-9} to 1.7×10^{-8} M. For each R6G concentration, 8–10 images were taken to ensure the accuracy of the results. After application of the threshold obtained using the above-mentioned method, the number of the bright spots in each image was counted. As shown in Figure 3, each image had a somewhat different number of molecules imaged even for the same concentration. However, the average number of bright spots in the images obtained for the same concentration has an excellent linear relationship with the R6G concentration. The correlation factor is 0.998. These results clearly demonstrate the single-molecule detection capability of our optical fiber probe based system. Using the data in Figure 3, the fraction of the molecules detected in our system is calculated to be about 6%.

To determine whether adsorption of R6G molecules on the fiber surface is significant, we replaced the R6G solution with water without rinsing the channel. We immediately took images and saw no significant increase in the number of bright spots in these images. This indicates that adsorption of the R6G molecules on the fiber surface is not severe. In addition, there are three other pieces of evidence to support this observation: First, the R6G concentration is so low that the surface adsorption is unlikely unless the optical fiber surface is specifically modified to do so. Second, the bright spots are larger for the R6G solution than those from water. If R6G molecules were adsorbed on the surface, the bright spots would then be on only one pixel since the R6G molecule was fixed. Third, consecutive images did not show fixed bright spots for several images in a row. We also carried out experiments to image R6G molecules in water (pH = 5.5) and in HCl aqueous solution (pH = 2); no difference was observed in these R6G images. All these clearly suggested that R6G molecules were not adsorbed on the optical fiber surface.

Plastic Square Fibers for Single-R6G Detection. A square fiber is a new type of plastic optic fiber. The core of the fiber is made of poly(methyl methacrylate) (PMMA), a polymer with superior optic properties. PMMA has a refractive index of 1.49. The cladding of the fiber is made of a fluoropolymer. Removal of the cladding is quite easy. A plastic square fiber is mainly used for light transportation within a short distance. This fiber is very attractive for SMD applications because of its flat core surface which is similar to that of an optical prism. In addition, plastic

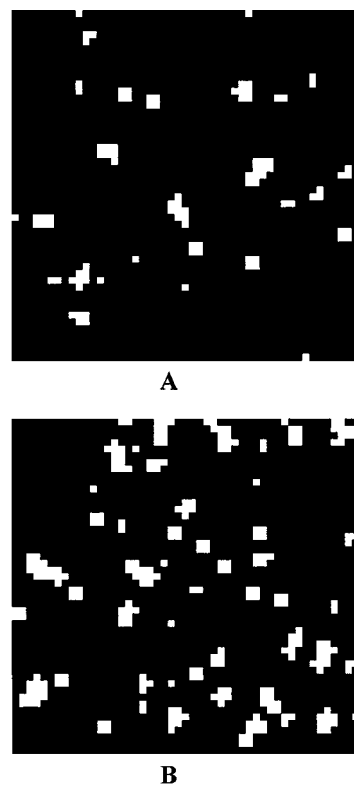


Figure 4. Fluorescent images (50×50 pixel subframe image) of single R6G-dUTP (B) and R6G (A) molecules. The concentrations of the samples were 1×10^{-8} M. A $365 \mu\text{m}$ core cylindrical fiber was used.

square fibers are much less expensive than the conventional fused-silica fibers. We have successfully imaged single R6G molecules (1×10^{-8} M) by using a plastic square fiber.

Dye-Labeled Biomolecule Detection. The optical fiber based SMD method has also been applied to the detection of dye-labeled biomolecules such as R6G-dUTP and BODIPY-avidin. Figure 4 shows an image of R6G-dUTP molecules. A comparison experiment between R6G molecules (Figure 4A) and R6G-dUTP molecules (Figure 4B) was performed when both concentrations were the same (1×10^{-8} M). We observed more bright spots for the R6G-dUTP solution than for the R6G solution. There are two possible reasons: First, this is in agreement with their relative quantum yields determined from their absorption and fluorescence spectra. The quantum yield for R6G-dUTP is 1.4 times higher than that for R6G under the same experimental conditions. Second, R6G-dUTP molecules remained within the evanescent wave field longer than R6G molecules. This is due to the slower diffusion of R6G-dUTP molecules than that of R6G molecules in an aqueous solution. A longer retention time for R6G-dUTP molecules staying within the evanescent wave excitation depth is thus expected during their random diffusion in the solution. Therefore, R6G-dUTP would be imaged with a higher probability and thus more bright spots. It also appears that these bright spots have larger sizes on average.

Images of single molecular beacon DNA molecules at different pHs have also been obtained as shown in Figure 5. Molecular beacons are a new class of single-stranded oligonucleotide probes which possess a stem-and-loop structure.²² They are potentially highly useful for DNA analysis and DNA sensors. The MB used here has 31 bases, of which 21 bases are the probe sequence and

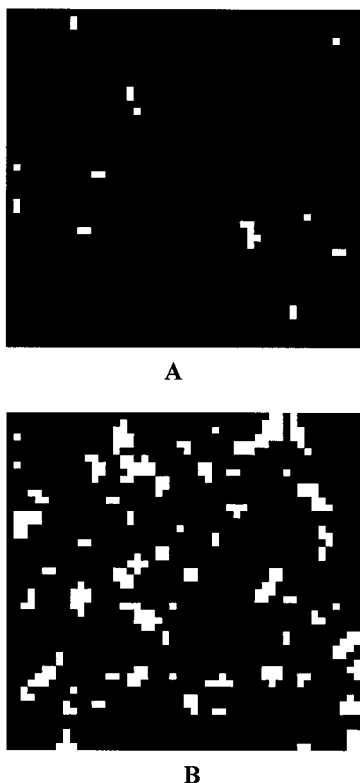


Figure 5. Fluorescent images (50×50 pixel subframe image) of single molecular beacon molecules at pH 8 (A) and pH 13 (B). The concentrations of molecular beacons were 1×10^{-8} M. A $365 \mu\text{m}$ core cylindrical fiber was used.

5 bases at each one of the two ends of the beacon are complementary to each other to form the stem. Tetramethylrhodamine (TMR) and 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) are linked to the two ends of the stem. Under normal conditions, where the stem keeps these two moieties in close proximity, the nonfluorescent DABCYL serves as a quencher of the fluorescence of TMR. As shown in Figure 5A, only a few bright spots are observed in a pH 8 buffer. Molecular beacons emit intense fluorescence when the stem is apart either through hybridization of DNA molecules with DNA molecules complementary to the loop sequence of the molecular beacon or by unwinding the stem hybrid by increasing the temperature or using a denaturing reagent. When a concentrated NaOH solution was added to change the molecular beacon solution (1×10^{-8} M) to a pH value of 13, the hydrogen bonds between the base pairs in the stem were broken, causing the fluorophore and the quencher to move away from each other. This led to the restoration of fluorescence of TMR. Therefore, many bright spots were observed in Figure 5B. As expected, the bright spots corresponding to single molecular beacon in the high-pH solution are relatively large due to slow diffusion within the evanescent wave field and the more intense fluorescence from the molecular beacons at higher pH.

Comparison between the Optical Fiber Approach and the Prism Approach. A prism has been used as a common and effective way to produce evanescent wave excitation for SMD. We conducted experiments with a glass prism using a similar method reported by Xu and Yeung.⁷ The results showed that an optical fiber was as efficient as a prism in single-molecule detection. There

are several advantages of using an optical fiber probe. For a prism, to achieve total internal reflection, the incident-ray angle must be carefully adjusted to be larger than the critical angle, which is determined by the refractive indexes of the two mediums at the interface. In the optical fiber based system, this alignment is not necessary. Once a laser beam is coupled to an optical fiber probe, an evanescent field is produced on the fiber core surface if the medium surrounding the core has a lower refractive index. An optical fiber has an obvious advantage in making the optical setup for SMD easier for experiments. Using an optical fiber probe, we are able to greatly reduce the number of optical components used to manipulate an optical beam and therefore simplify the optical arrangement for the transportation of light. The probe is more flexible for use in different geometries as needed to meet the requirements of each analysis. An optical fiber is also capable of transporting light over a long distance without much light loss and without using even a single mirror or a focusing lens. Moreover, as there are many well-developed techniques for detecting biomolecules and studying their interactions using fiber-optic sensor and evanescent sensors,^{17-21,26} we thus expect that the present SMD technique will benefit from this rich database for practical applications. The convenience in generating an evanescent field on an optical fiber surface will make SMD more accessible and surely enable many practical applications of SMD. The work being undertaken in our laboratory is directed toward using a biomolecule-immobilized optical fiber probe for single-molecule interaction studies. This probe might also be useful as a remote sampling probe to capture single-molecules in real-life samples; the fiber can then be imaged through the SMD technique developed in this work. We believe that the optical fiber will be an effective probe for efficient applications of SMD techniques.

CONCLUSION

We have developed a convenient yet effective method for the optical detection of single fluorescent molecules. An optical fiber probe was used to produce an intense evanescent wave for SMD. An optical fiber based microscope system coupled with ICCD detection was built for SMD in solution and at the interface of an optical fiber probe. Single dye molecules and dye-labeled biomolecules were imaged using two different types of optical fiber probes, cylindrical fiber and plastic square fiber. An excellent linear relationship between the fluorophore concentration and the number of molecules imaged at the interface was obtained. The method developed in this work is simple, convenient, and easy to implement for practical applications of SMD. Our single-molecule imaging capability will be explored for single-molecule sensor applications and for studies of single-molecule interactions and reactions at interfaces.

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