Application Note

Single molecule imaging and interaction study using evanescent wave excitation

BY XIAOHONG FANG AND WEIHONG TAN

RECENTLY, THERE HAVE been important advances in applying fluorescence techniques for the observation of species at the level of single molecules and the study of their behaviors.[1,2] In order to realize many features important for biological systems, single molecule detection (SMD) in physiological relevant environments, such as in solution, is of increasing interest.[3–12] The challenge for the detection of single fluorescent molecules in solution is to extract signals from a high background due to light scattering and fluorescent impurities.[2] One of the most effective ways to increase the signal-to-noise ratio for SMD is to use evanescent wave excitation.

Evanescent wave is produced by total internal reflection at an interface. Its intensity decays exponentially from the interface, resulting in a penetration depth usually less than half of the excitation wavelength. Use of an optical prism-based system as the waveguide is a common way to generate an evanescent field.[4–7] Recently, a simple method for imaging single fluorescent molecules using evanescent wave produced by an optic fiber was developed.[8] The optical fiber-based SMD technique is easy and is as effective as the prism system; it can benefit from the rich database of fiber optic biosensor technology. The scheme of using evanescent wave to excite single fluorescent molecules, either by a prism or an optical fiber, is being explored for the study of single molecule reaction and interaction. This paper discusses the single molecule study using an optical fiber probe.

Experimental

Figure 1 schematically depicts the setup for the detection of single molecules in solution. The system consists mainly of a microscope, an intensified charge-coupled device (ICCD), argon ion laser, and optical fiber probe or prism. The microscope was equipped with a 100× objective (LM plan F1, 0.8 NA [numerical aperture], **Olympus** [Melville, NY]). An ICCD (EEV 512 × 1024 FT [frame transfer], **Roper Scientific, Inc.** [Trenton, NJ]) was mounted on the top entrance port of the microscope. Excitation laser beams were produced from an Innova 307 Ar^+ laser (**Coherent Laser,** Santa Clara, CA). For the optical fiber setup, the laser beam was directed to one end of the optical fiber probe. The other end of the fiber was inserted into a sample channel, which was placed on the stage of the microscope and filled with a sample solution. Evanescent field was generated on the surface of a section of exposed core area on an optic 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, either a 530-nm long pass filter and a 550-nm interference filter were placed in front of the ICCD camera for the detection of rhodamine 6R (R6G) molecules, or two optical filters (550 and 570 nm) for the detection of molecular beacon (MB) DNA molecules, which are labeled with tetramethylrhodamine (TMR). Images were taken using a subframe of 50 × 50 pixels. The exposure times used for the collection of images of single molecules were 30–100 msec.

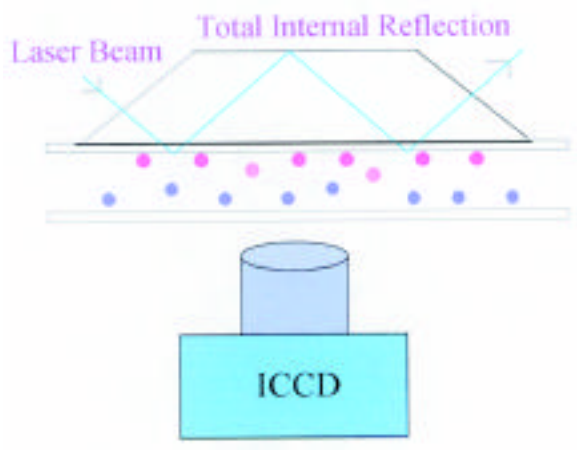

Figure 1 *Instrumental setup for single molecule detection and imaging in solution using evanescent wave excitation.*

Results and discussion

Evanescent wave excitation for single molecule detection

The evanescent field has a small penetration depth in the vertical direction and thus only excites the fluorophores exclusively in the close proximity of the interface. This greatly reduces the background signal from the bulk solution, minimizing the detection volume and enabling investigation of surface phenomena. Since the depth of the evanescent field in the authors' experiment is about 250 nm, and the pixel size of the ICCD camera is 23 µm, they estimated that one pixel of the ICCD only detects a volume in the 10^{-17} L range when a 100× objective is used. This is a small enough volume to isolate individual molecules, even with a fluorophore concentration in the 10^{-7} *M* range. When more diluted fluorophore concentrations are used, the probability of detecting two or more molecules at the same pixel is negligibly low. This is one of the important considerations in realizing SMD.[7]

Imaging one molecule at a time

Both conventional cylindrical fibers and a new type of polymethylmethacrylate (PMMA) square fibers have been used for the detection and imaging of single dye molecules, fluorescein and R6G, and some dye labeled biomolecules.[8] *Figure 2* shows the images of pure water and individual R6G molecules using a square fiber. The image obtained for water was used to determine the threshold for single R6G images. More bright spots were shown in the images for the R6G solution (Figure 2*b*). Each bright spot represented one R6G molecule 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.

There are three observations to support that single R6G molecules were indeed imaged. First, statistically, the detection volume and the R6G concentration enabled at most only one molecule at each pixel, with the vast majority of the pixels without any molecules. Second, the number of single molecules imaged in the

Figure 2 *Fluorescent image (50 × 50 pixel subframe image) of single R6G molecules in water. A square fiber was used. a)Purified water. b) 1.7×10^{-8}* M *R6G solution.*

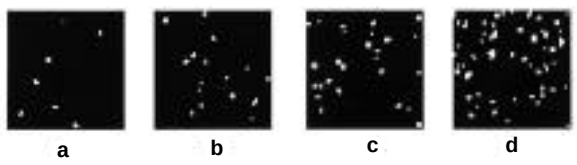

Figure 3 *Fluorescent images (50 × 50 pixel subframe image) of single molecular beacon DNA molecules at pH 8 (20 m*M *tris/HCl, 50 m*M *KCl, and 5 m*M *$MgCl_2$) after interaction with their complementary DNA molecules for 0 min (a), 2 min (b), 8 min (c), and 20 min (d).*

authors'experiments were in close agreement with the R6G concentration used, taking account of some 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.[8]

Monitoring single molecule reactions and interactions

The authors are exploring the single molecule imaging capability for the studies of single molecule interactions. *Figure 3* shows an example of imaging single molecular beacon DNA molecules during hybridization. Molecular beacons are a new class of single-stranded oligonucleotide probes used for DNA analysis and DNA sensors. They have a stem-and-loop structure with a fluorophore and a quencher linked to both ends of the stem.[13–15] The molecular beacon used in this experiment is 5-(TMR)CCT AGC TCT AAA TCG CTA TGG TCG CGC TAG G(_DABCYL)-3′.[15] The concentration of the MB is low so that each pixel of the ICCD detects only one MB molecule. Under normal conditions, as the stem keeps these two moieties in close proximity to each other, the fluorescence of TMR is quenched. Only a few bright spots are observed (Figure 3*a*). These bright spots correspond to single MBs that are not totally fluorescent quenched. The molecular beacons emit intense fluorescence when the stem is apart through hybridization of DNAmolecules complementary to their loop sequence. This led to the restoration of fluorescence of TMR. Therefore, the number of bright spots increased with the hybridization time (Figure 3*b–d*).

These results demonstrate that using evanescent wave excitation is an effective method for SMD and can be applied to the study of biomolecules in physiological relevant environments. Potential applica-

tions that are unique to single molecule probing include investigating nanometer scale environments, monitoring individual molecule reactivity, studying the variability of molecular conformations, devising nanostructures, and single molecular-scale imaging probes for scanning probe microscopy. It should greatly improve our understanding of the elementary steps in biochemical reactions and life processes as well as the development of sensitive monitoring technologies for biochemical species. Better comprehension of these interactions is expected to have great implications in understanding biomolecular functions and their diagnostic applications in medicine, in studying disease mechanisms, in designing new drugs, and in the development of functionalized nanomaterials for a variety of novel applications.

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The authors are with the Dept. of Chemistry and UF Brain Institute, University of Florida, Gainesville, FL 32601, U.S.A.; tel.: 352-846-2410; fax: 352-392-4651; e-mail: tan@chem.ufl.edu.