# Ultra-Sensitive Real-Time Single-DNA Molecules Detection at a Fused-Silica/Water Interface Using TIRFM Technique

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*Received February 21, 2005*

**Key Words :** Single molecule detection, Microchip, TIRFM, Ultra-sensitive

Many scientific results are based on macroscopic observations of group behavior of large numbers of molecules. Recent single molecule detection (SMD) techniques in free solution have allowed for the direct observation and manipulation of single biomolecules, which permitted their dynamic behaviors and localization to be studied to provide insight into molecular genetics,[1-3] biochip assembly,[4-8] biosensor design,[9-11] DNA biophysics[12-21] and basic separation theories.[22-25] However, the major problem to overcome with individual biomolecules in an aqueous solution is the huge amount of background noise, which can be caused by Raman scattering from water molecules, incident light, luminescence from the objective lens, and dust.[26] The main approach to reduce the background noise and improve the signal-to-noise ratio is to keep the detection volume as small as possible. This is because the noise decreases proportionally to the detection volume, whereas the signal from single molecules is independent of the detection volume.[27] In general, a volume of 10 pL or less is used for the SMD.[28] In a special case, the detection volume of the order of femtoliters can be reached using a confocal fluorescence microscope.[18,29] However, although the confocal microscopy is sensitive enough in a static condition, the molecular detection efficiency in a driving condition is extremely low.[30] Meaningful results can be obtained at the concentration range of $10^{-9}$ to $10^{-10}$ M.[27] Another simple approach to increase the molecule detection efficiency with a conventional volume of nL-$\mu$L is to use high power lasers to produce high fluxes of photons from the molecules labeled with fluorophore. Large DNA or protein molecules can be labeled with many fluorophores to further facilitate detection. However, in this case of using a high power laser, the intensity from the incident laser beam must be decreased to minimize the noise value of the signal-to-noise ratio.

Total internal reflection fluorescence microscope (TIRFM) optics is significantly more sensitive to the small fluctuations and/or background noise than conventional epifluorescence microscopy. After single fluorophores in an aqueous solution were first observed in 1995 using TIRFM and conventional inverted fluorescence microscopy,[17] some biophysicists and chemists have used the TIRFM technique for many years, while others are just beginning to explore the boundaries of this versatile mechanism for studying phenomena occurring at interfaces. Briefly, the total internal reflection (TIR) of visible light at a solid/liquid interface produces a region of optical intensity, evanescent waves that penetrate just 150-200 nm into the liquid phase. This interfacially constrained optical field is the basis of all TIR and attenuated total reflection types of spectroscopy and microscopy. In principle, the only DNA molecules observed under TIRF optics are those that are intrinsically or extrinsically fluorescent, and that lie close enough to the solid/liquid interface to be within the region illuminated by the evanescent wave. This means that individual DNA molecules that enter the evanescent field can be seen as separated, diffraction-limit spots, and that background fluorescence signals from out-of-focus DNA molecule labeled with fluorescent dyes is minimized.

In our experiments, we wish to observe attomolar single DNA molecules moving on the surface with real-time. This work was focused on ultra-sensitive ($10^{-17}$-$10^{-18}$ M) real-time single DNA molecule detection at a fused-silica/water interface using a home-built TIRFM system.[4] To achieve this, we expressed them as adsorption/desorption at different pH levels with individual DNA molecules labeled with fluorescent dye YOYO-1 for excitation by an argon ion laser that was focused through the evanescent field layer. An intensified CCD camera was used to image the individual DNA molecules with a real-time of 0.2 ms time resolution. When the light beam was reflected in the TIR region, an evanescent field layer was generated at the interface of water and the fused-silica prism surface. Because the evanescent field was restricted to the region of the interface, the DNA molecules were excited only as they entered the field.

The fluorescence images for 50 aM of $\lambda$-DNA molecules (48,502-bp) at different pHs in the fused-silica/water interface by home-built TIRFM and conventional epifluorescence microscope are shown in Figure 1. The real-time conformation change of individual DNA molecules was not observed in the epifluorescence microscope (Figure 1B), but only in the TIRFM (Figure 1A) due to photo-blenching. At pH 8.2 in the TIRFM, individual DNA molecules resembled random coils (Figure 1A). They were constantly moving between exposures. At pH 6, DNA molecules started to be adsorbed onto the prism surface. l-DNA molecules showed a linear combing phenomenon in the pH range of 4-6. It is well known that both electrostatic and hydrophobic inter-

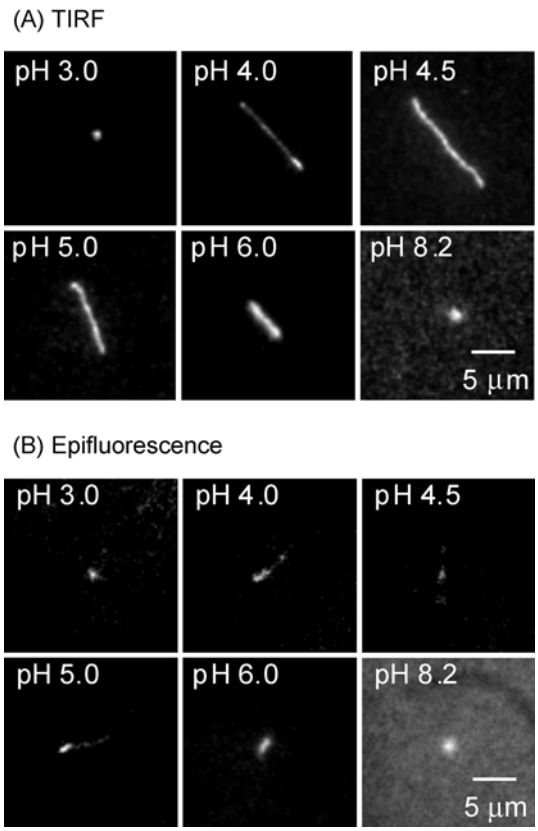


**Figure 1**. TIRFM (A) and epifluorescence (B) images of $\lambda$-DNA molecules labeled with a fluorescent dye YOYO-1 at a ratio of one dye molecule per five base pairs at different levels of pH. TIRFM experiment conditions: DNA sample concentration, 50 aM; sample volume, 250-nL on a glass cover slip (10 mm square); camera, ICCD Pentamax 512-EFT/1EIA; ICCD exposure time, 10 ms; objective, 100 ×/1.3 NA Plan-Neofluar; laser out power, 25 mW. Epifluorescence experiment conditions: microscope, Zeiss Axioskop 2 upright; light source, 100 W mercury lamp; filter set, a Zeiss filter set No. 9. Others epifluorescence experiment conditions were the same as those in the TIRFM.

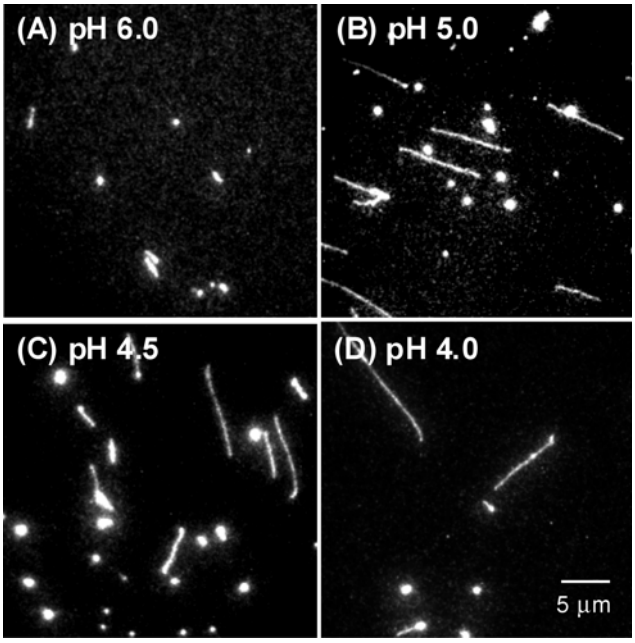


**Figure 2**. Video TIRFM fluorescence images of a mixture of circular ΦX174-DNA molecules (5,386 bp, circle form) and $\lambda$-DNA molecules (48,502 bp, linear form) at 50 mM sodium acetate buffer of (A) pH 6.0, (B) pH 5.0, (C) pH 4.5 and (D) pH 4.0. DNA sample concentration, 250 fM; sample volume, 250 nL. Other experiment conditions were the same as those in Figure 1.

actions govern DNA and protein adsorption at liquid/solid interafces.[9,20,24] However, this result contrasts a previous report that l-DNA molecules started to adsorb onto the fused-silica prism surface at near pH 5.5.[24]

The fluorescence images of the mixture of different size DNA molecules (*i.e.*, 48,502-bp and 5,386-bp) at different pHs in the interface by TIRFM are shown in Figure 2. Individual ΦX174-DNA molecules still exhibited circular form because the original shape of ΦX174-DNA is circular (small circular spots in Figure 2). These results indicate that the thickness of a sample chamber for single-DNA molecule experiments can affect the pH which starts the adsorption onto the prism surface. The thickness of the sample solution from a previous experiment was ~8 $\mu$m,[24,25] but now the thickness was ~2.5 $\mu$m. Below pH 3.0, all DNA molecules were completely adsorbed in the compact form. These results correlate with previous results except the staring pH to adsorb.[24] Since the measurement only depends on being able to follow a molecule for a few milliseconds in the solution, the different size DNA molecules could be analyzed within a few milliseconds at an optimum pH, such as the starting pH to show linear combing (Figure 2B).

In our TIRFM set up, we could also observe the image of individual 100-bp single-DNA molecule with 50 aM concentration (Figure 3A). A concentration of $5 \times 10^{-17}$ M means there exists only a few molecules in each 250-nL volume, or a $1 \times 1 \times 2.5 \times 10^{-7}$ mm$^3$ region. The detection window of TIRFM is $9.5 \times 10^{-3}$ mm$^2$. In this small detection volume and ultra-low concentration, the probability of finding one molecule is $9.5 \times 10^{-5}$. However we have shown in this experiment that nearly 100% molecular detection efficiency can be achieved with our TIRFM system.

To summarize, we demonstrated direct observation of attomolar DNA sample concentration at a fused-silica/water interface by counting individual DNA molecules. The TIRFM technique was improved to facilitate DNA molecule unity. This TIRFM technique could be used for analyzing different size DNA molecules within a few milliseconds based on characterizing the dynamics and shapes of individual DNA molecules changed with each pH. The ability to detect individual DNA molecules at solid/liquid interfaces with real-time should increase the capabilities of researchers studying the basic interactions between a nanoarray DNA biochip. Although we have explored only a fused-silica prism surface, we expect that it can be extended to a wide range of biochip substrates patterned with various biological molecules, such as DNA, proteins, and cells.

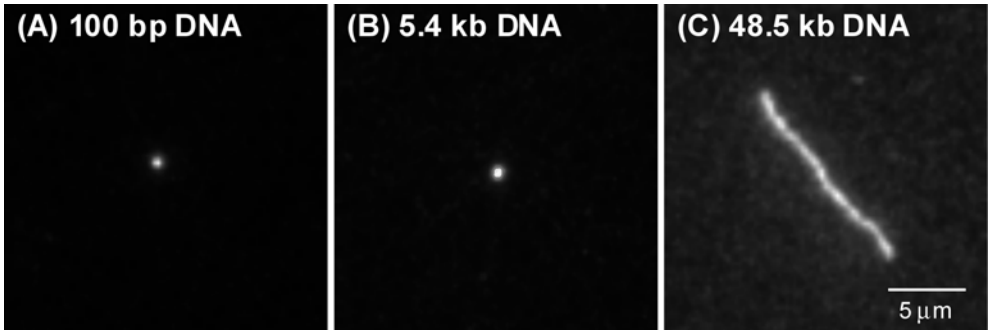


**Figure 3**. Real-time TIRFM fluorescence images of (A) 100 bp-DNA, (B) circular ΦX174-DNA molecules (5,386 bp-DNA) and (C) $\lambda$-DNA molecules (48,502 bp-DNA) labeled with fluorescent dye YOYO-1 at 50 mM sodium acetate/acetic acid (pH 4.5). The DNA sample concentration was 50 aM. Other experiment conditions were the same as those in Figure 1.

## Experimental Section

**Chemicals.** The various buffer systems used are as follows: sodium acetate/glacial acetic acid (pH 4-6) and acetic acid sodium citrate/citric acid (pH 3). Sodium acetate, glacial acetic acid, sodium citrate, citric acid and sodium hydroxide were ACS grade purchased from Sigma Chemical Co. (St. Louis, MO, USA). A 10 mM aqueous solution of Gly-Gly (Sigma Chemical Co., St. Louis, MO, USA) was prepared and the pH was adjusted to 8.2 with the addition of 2 M NaOH. All buffers were filtered through a 0.2-$\mu$m filter and left for photo-bleaching overnight with a UV-B lamp (G15T8E, 280-315 nm, Hansung Ultraviolet Co, Ltd., Korea) prior to use.

**DNA Samples Preparation.** $\lambda$-DNA (48,502 bp) and ΦX174-DNA (5,386 bp) were obtained from Promega (Madison, WI, USA). All DNA samples were prepared in the photobleached Gly-Gly buffer described above. YOYO-1 was diluted 100 times with Gly-Gly buffer and then mixed directly with DNA. DNA samples were labeled with a fluorescent dye YOYO-1 (Molecular Probes, Eugene, OR, USA) at a ratio of 1 dye molecule/5 base pair. DNA samples were prepared at a concentration of 200 pM. The samples were allowed to incubate for 5 min before further dilution and use. For the single DNA molecule imaging experiments, the DNA samples were further diluted to 50 aM-500 fM with the prepared pH buffers just prior to the start of the experiment.

**Evanescent Wave Excitation Geometry.** The excitation geometry was similar to that previously reported.[24,25] The sample chamber for single-DNA molecule experiments was constructed by sandwiching a 250-nL volume of DNA sample between a No. 1 (10 mm square, refraction index (n) = 1.523) Corning glass cover slip and the hypotenuse face of a right-angle fused-silica prism (Melles Griot, Irvine, CA, USA; prism UVGSFS, A = B = C = 25.4 mm, n = 1.463). A focused argon ion laser beam was directed through the prism toward the prism/sample interface. The incident angle $\grave{e}_i$ was greater than the critical angle $\theta_c$ defined by Snell's law. In this system, $\theta_i$ was about 68°.

**Microscope and ICCD Camera.** A Pentamax 512-EFT/1EIA intensified CCD (ICCD, Princeton Instruments, Princeton, NJ) camera was mounted on top of a Zeiss Axioskop 2 upright microscope with a Zeiss 100 × Plan-Neofluar oil type objective (1.30 NA). The objective was optically coupled to the cover slip with immersion oil (Immersol$^{TM}$ 518F, Zeiss, n = 1.518). The digitization rate of the camera was 5 MHz with the software controller gain set at 3 and hardware intensifier gain set at 80. The camera was operated in the external synchronous mode with the intensifier disabled open and was also used in the frame-transfer mode. A 488-nm argon ion laser (532-AP-A01, Melles Griot, Irvin, CA, USA) was used as the excitation beam. A Zeiss Filter set No. 09 was used. A Uniblitz mechanical shutter (model LS2Z2, Vincebnt Associates, Rochester, NY, USA) was used to block the laser beam when the camera was off to reduce the photobleaching of DNA samples. The shutter was controlled by a model VMM-D1 shutter driver (Vincent Associates, Rochester, NY, USA) with a frame rate of 10 Hz.

**Single-Molecule Timing.** The experimental timing was controlled with a Stanford Research Systems model DG535 four-channel digital delay/pulse generator (Stanford Research Systems, Inc., Sunnyvale, CA, USA). The ICCD camera was triggered at a time of 0-ms with 10-ms duration TTL pulse. The sampling frequency was 10 Hz, with the shutter driver set to 10-ms exposure and 90-ms delay. The WinView/32 software (version 2.5.14.1, Downingtown, PA, USA) was used for DNA image collection and data processing.

**Acknowledgements.** The authors wish to thank the research team of the Center for University-Wide Research Facilities (Chonbuk Natl. Univ.) for their assistance with instrumentation. This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG4-PT02-0042).

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