**Partial Sequencing of a Single DNA Molecule with a Scanning Tunnelling Microscope**
Paper in journals : this is the first page of a paper published in ***Nature Nanotechnology*** .
[***Nature Nanotechnology***] **4**, 518-522 (2009)

LETTERS
PUBLISHED ONLINE: 5 JULY 2009 | DOI: 10.1038/NNANO.2009.155
nature nanotechnology

## Partial sequencing of a single DNA molecule with a scanning tunnelling microscope

Hiroyuki Tanaka* and Tomoji Kawai*

**The scanning tunnelling microscope is capable of the real-space imaging and spectroscopy of molecules on an atomic scale. Numerous attempts have been made to use the scanning tunnelling microscope to sequence single DNA molecules, but difficulties in preparing samples of long-chain DNA molecules on surfaces, and problems in reproducing results have limited these experiments[1–6]. Here, we report single-molecule DNA sequencing with a scanning tunnelling microscope by using an oblique pulse-injection method to deposit the molecules onto a copper surface. First, we show that guanine bases have a distinct electronic state that allows them to be distinguished from the other nucleic acid bases. Then, by comparing data on M13mp18, a single-stranded phage DNA, with a known base sequence[7], the 'electronic fingerprint' of guanine bases in the DNA molecule is identified. These results show that it is possible to sequence individual guanine bases in real long-chain DNA molecules with high-resolution scanning tunnelling microscope imaging and spectroscopy.**

We have previously detected the characteristic electronic states of the fluorescent dye molecule fluorescein isothiocyanate (FITC) in single-molecule scanning tunnelling microscope (STM) observations and $I-V$ measurements of short strands of DNA and peptide nucleic acid (PNA) labelled with FITC[8], but we could not observe peaks in the characteristic electronic states of base molecules due to scatter and the poor signal-to-noise ratio of the $I-V$ curve[9–11]. Here, by using lock-in detection[8], we have successfully detected electronic states that are exclusively characteristic of DNA/PNA.

Figure 1 shows typical STM images of single-stranded monomers of FITC-modified PNA (FITC–TTGACC) and DNA (FITC–TTGGCC), as well as the spectroscopic data for these samples. As can be seen from the wide-area images (Fig. 1a,d), it is difficult to fully determine the base sequences of oligomers due to the existence of other adsorbed materials (thought to be nucleotide monomers, buffers and salts that were not completely eliminated in the purification process) and the diversity of the adsorbed structures of the oligomers themselves. However, by comparing two types of oligomer, it is possible to assign guanine. With FITC–TTGACC, only one base is seen to shine in the middle of the base sequence, whereas with FITC–TTGGCC there are two, as can be seen from the wide-area image (Fig. 1d) and enlarged image (Fig. 1e). Furthermore, with tunnelling spectroscopy obtained by lock-in detection, it can be seen that the guanine base molecules differ from the other base molecules and the underlying substrate in that they have a characteristic peak in the electronic state in the vicinity of an applied voltage ($V_s$) of −1.6 V (Fig. 1c,f). Of the four bases, it is thought that guanine is the easiest to detect because it has the lowest ionization potential[12]. To the best of our knowledge, there are no previous STM reports where the characteristic state density of base molecules, adsorbed onto a metal substrate, have been detected in this way[4,9–11,13,14].

Recent theoretical calculations[15] of DNA base molecules adsorbed on a Cu(111) surface suggest that peaks in the density of states capable of being measured by STM should exist for all four types of base molecules and not just guanine. Although the calculated energy of the first peak below the Fermi energy of the guanine/copper agrees well with our experimental values, the theoretical predictions for the other bases are inconsistent with our STM measurements. It is not understood why this characteristic electronic state occurs only for guanine bases. However, to determine if these results are also correct for real DNA, and if they are effective for sequencing, we analysed long-chain DNA strands.

The difficulties encountered in the past in sequencing single long-chain DNA molecules with the STM have been due, in part, to the unsuitability of the sample preparation methods[2]. Although researchers have managed to observe the macro structure of DNA[3,4], few studies have been able to make use of the full resolving power of the STM, that is, the clear and reproducible observation of individual nucleotides. Detailed STM imaging, spectroscopy and manipulation studies of molecules are almost always restricted to molecules that can be deposited onto a surface from the gas phase[16,17], although alternative sample preparation methods have been examined[1,18–20]. By developing a pulse-injection technique for depositing molecules onto substrates that cannot be vapour deposited (including supramolecular and biological molecules such as DNA), we have previously achieved reliable and repeatable high-resolution STM observations of the helical period of circular double-stranded (plasmid) DNA, and of the individual nucleotides in single-stranded (ss) DNA oligomers and DNA polymers[1,5,6]. However, this technique suffers from the fact that ssDNA can easily form secondary structures due to intrastrand base-pairing, with the result that sections of sufficient length to allow sequencing are rarely observed[6]. Most of the DNA stretching methods that have so far been developed for ascertaining the dynamics of DNA and analysing single-molecule genes are either performed in liquid[21] or atmospheric conditions[22], making them unsuitable for high-resolution, ultrahigh-vacuum studies. It was therefore necessary to develop a technique for stretching out ssDNA strands.

We have developed[23] a method for stretching out and fixing single-stranded M13mp18 DNA molecules (which contains 7,249 bases)[7] by using the flow effects resulting from the oblique injection[8] of a DNA solution onto a substrate using a pulse-injection technique (Fig. 2a). When the DNA solution is injected perpendicularly onto the substrate, no extended DNA is observed (see Supplementary Fig. S1). Figure 2b shows a typical wide-region STM image (400 nm wide) of an ss M13mp18 extended and fixed by oblique injection on a Cu(111) surface. Owing to the height differences at steps in the substrate, the contrast is not ideal for recognizing DNA molecules, but it is possible to recognize two DNA strands running from left to right. Figure 3 shows the image obtained over a wider area (800 nm wide). As a result of the wide scan

The Institute of Scientific and Industrial Research, Osaka University, Mihogaoka, Ibaraki, Osaka 567-0047, Japan. *e-mail: hrtanaka@sanken.osaka-u.ac.jp; kawai@sanken.osaka-u.ac.jp



▲Reprinted with permission from ***Nature Nanotechnology,*** 4, 518-522(2009). Copyright 2009 Nature Publishing Group.

The following is a comment on the published paper shown on the preceding page.

# Partial Sequencing of a Single DNA Molecule with a Scanning Tunnelling Microscope

***TANAKA Hiroyuki and KAWAI Tomoji***
(The Institute of Scientific and Industrial Research)

## Introduction

Although developments in science and technology have resulted in higher standards of living, people continue to strive for a safer society with access to personal medicine. Investigations aimed at the development of a high-speed, cheap, single-molecule-based sequencer have been performed worldwide using advanced technologies such as nanotechnology and biotechnology. Since the development of scanning tunnelling microscopy (STM), a typical technological tool that allows for the visualization and manipulation of individual molecules and atoms, researchers have attempted to sequence individual nucleotides in DNA using STM. Although several STM images showing the apparent macrostructure of DNA were reported by researchers,[1] it was concluded at about 1990 that the majority of the images represented nothing but artifacts.[2] Notwithstanding the continued efforts of researchers devoted to the STM imaging of DNA or DNA-related molecules, the general consensus seemed to suggest that it is impossible to realize high resolution STM imaging and sequencing of macromolecules such as DNA.

It became clear that one of the difficulties concerned the sample preparation method. STM achieves highest resolution under vacuum conditions. Under other conditions such as in air or liquid, the resolution of the image is easily lost by contamination. DNA molecules require pH-controlling buffer solutions, the chemical components of which (salts and buffer molecules) can lead to disturbances in high resolution STM observations. Since most of the salts and buffer can be removed by dialysis, DNA is typically deposited directly onto a surface from solution under ambient conditions and the sample is then loaded into the vacuum STM chamber. This method, however, does not provide a sufficiently clean substrate to allow for high-resolution imaging. If concentrated residual salts and buffer cover the DNA during the drying process, this can lead to additional contamination.

In an effort to overcome the aforementioned difficulties, we developed our own deposition method and became the first group to successfully achieve the high-resolution STM imaging of DNA.[3-5] In our deposition method, the DNA solution is injected directly into an ultrahigh vacuum using a pulse valve directed at a surface. Although both DNA and solvent water molecules adsorb onto the surface, the water molecules evaporate from the surface before appreciable aggregation of DNA occurs. Use of this method also minimizes the aggregation of salts and buffer onto DNA strands and therefore improves high-resolution STM imaging. It should be noted that injection of the amounts of water employed into an ultrahigh vacuum (UHV) chamber may result in a fatal crash of the expensive UHV pump system, in addition to contamination of the valuable substrate and vacuum chamber; the use of conventional UHV experiments should obviate such outcomes.

We made great strides in visualizing individual nucleotides within a DNA oligomer, in addition to imaging the macrostructure of a DNA oligomer and supramolecules. However, due to the intrastrand base-pairing within long-chain single-stranded DNAs (ssDNAs), the macromolecules are not sufficiently stretched out on the surface to facilitate efficient and reliable sequencing. Most of the DNA stretching methods that have been developed to date for ascertaining the dynamics of DNA and analyzing single-molecule genes are either performed in liquid or atmospheric conditions, making them unsuitable for high-resolution, ultrahigh-vacuum studies. It was therefore necessary that a technique be developed for stretching out ssDNA strands.

Fortunately, we recently overcame this problem by modifying our previously developed deposition method. With this new approach, the DNA solution is injected at a tilted surface.[6] This method stretches out the individual DNA molecules across the surface and allows the molecules to be imaged without removing them from the vacuum. We found that when measuring the conductance spectrum (the derivative of the current–voltage spectrum) over the four different DNA bases, guanine showed a characteristic conductance peak when the STM tip was biased at −1.6 V. This characteristic is unique to guanine. By imaging a stretched, single-stranded M13mp18 DNA molecule,[7] with the sample biased at this voltage, we obtained an image in which the guanine bases appeared larger and brighter, and therefore could readily be identified. By matching the relative position of the guanine bases with the known sequence, we were able to sequence the guanine bases and identify the imaged segment within the known sequence of a real long-chain DNA molecule.

## Experimental procedure

We used two low-temperature STM systems, one (LT–STM, Omicron GmbH) and the other (USM-1200S2N1, Unisoku). Both STM chambers were cooled by liquid nitrogen, and the observation temperature was 80 K. The substrate used was a Cu(111) surface, cleaned to be atomically flat by argon ion sputtering at 773 K in ultrahigh vacuum. M13mp18 ssDNA was purchased from Bayou Biolabs and was subjected to dialysis (Slide-A-Lyzer MINI Dialysis Unit, 10,000-molecular-weight-cut-off type; Rockford) against water to remove excess buffer solution and salt. M13 mp18 DNA was dissolved in water at concentrations of 0.5 nmol $l^{-1}$ and was deposited on clean Cu(111) surfaces using the oblique pulse injection method at room temperature(see Fig.1a).

The distance from the pulse valve to the substrate was 50 mm.