DNA Sequencing

DOI: 10.1002/anie.200503154

DNA Sequencing Based on Intrinsic Molecular Charges**

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Gene functional analyses have proceeded remarkably in the fields of molecular biology, pharmacogenomics, and clinical research, on the basis of completion of the decoding of the human genome. The analysis of nucleotide variation has become increasingly important for the assembly of a high-resolution map of disease-related loci and for clinical diagnostics. The most common form of genomic variation is single-nucleotide polymorphism (SNP), which is an important marker in personalized medicine that affects disease susceptibility and resistance. Although a number of methods for SNP analysis have been developed,[1–7] DNA sequencing techniques still need to be improved in terms of cost, simplicity, and throughput to analyze not only SNPs but also genomic variations, such as insertion/deletion and short tandem repeats.

We have been investigating a new approach to the direct, simple, and highly sensitive detection of nonlabeled molecular recognition events on a miniaturized and arrayed solid-state device.[8] Recently, several types of field-effect devices have been used for the electrochemical detection of hybridization events on a solid surface.[9] As DNA molecules are negatively charged in an aqueous solution, the number of negative charges at the gate surface of field-effect devices increases as a result of hybridization and extension reactions. The charge-density change is directly transduced into an electrical signal by the field effect. Based on this principle, point-mutation analysis was carried out by using the PCR products amplified with allele-specific primers.[9e] In this case, the overall specificity was determined from that of the allele-specific PCR. A single-base mismatch could also be distinguished by hybridization with complementary and mismatched DNA probes immobilized on the capacitor-type field-effect devices.[9b]

We propose a new method for DNA sequencing of known as well as unknown sequence variants, which is based on detection of the intrinsic charges of DNA molecules by using the field effect. Herein, we report the direct transduction of single-base extension at the gate surface into an electrical signal, and the possibility of label-free DNA sequencing based on the intrinsic charges of DNA molecules.

Oligonucleotide probes are immobilized on the Si_3N_4 gate surface, and the complementary target DNA is hybridized with these probes. The hybridization events are followed by the introduction of DNA polymerase and one of each deoxynucleotide (dCTP, dATP, dGTP, or dTTP). DNA polymerase extends the immobilized oligonucleotide probes in a template-dependent manner (Figure 1). As a result of the extension reaction, the number of negative charges increases at the gate surface of the field-effect transistor (FET) because of the intrinsic negative charges of the incorporated molecules. This change in charge density can be detected as a shift in the threshold voltage (V_T) of the FET. Thus, iterative addition of each deoxynucleotide and measurement of the threshold voltage allow direct, simple, and label-free DNA sequencing.

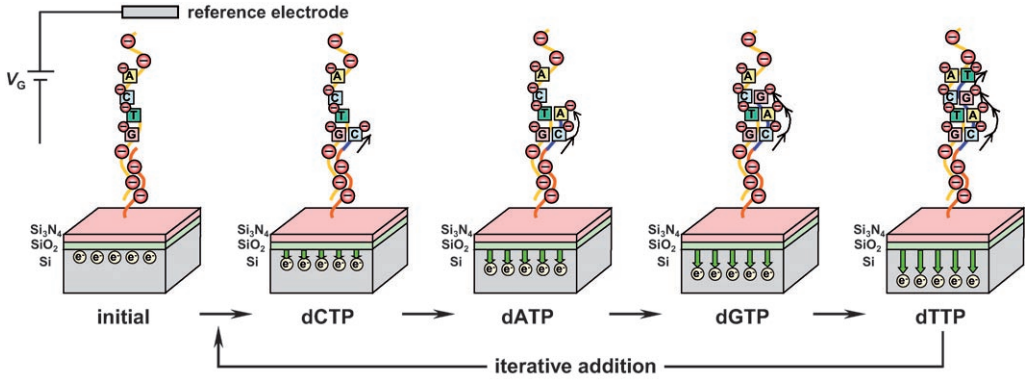

Figure 1. DNA sequencing based on FETs in combination with an extension reaction. Each deoxynucleotide is incorporated into the probe–target duplex on the FET in the following order: dCTP, dATP, dGTP, and dTTP.

The FET chip (Figure 2A) is immersed in a measurement solution together with an Ag/AgCl reference electrode with saturated KCl solution. The potential of the measurement solution is controlled and fixed by the gate voltage (V_G) through the reference electrode (Figure 2B). The base sequences of the factor VII gene, which include two SNP sites, and of the hereditary hemochromatosis gene

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[**] We thank Dr. Y. Horiike, Dr. J. Tanaka, Dr. H. Otsuka, and A. Ueda of the National Institute for Materials Science, Japan, Y. Nakajima of Ryokusei M.E.S. Co., Ltd., Japan, M. Kamahori of Hitachi Ltd., Japan, and Dr. P. Fortina of Thomas Jefferson University, USA, for their help and useful discussions.

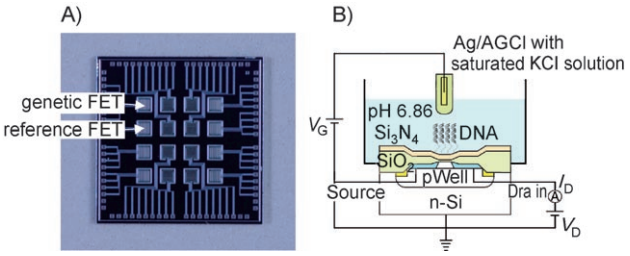

Figure 2. A) Photograph of the fabricated FET chip. Sixteen FETs and a temperature sensor are integrated in a 5×5-mm^2 chip. The FETs are of the n-channel depletion type with Si_3N_4/SiO_2 as a gate insulator. Two FETs were used for DNA sequencing: one FET with immobilized oligonucleotide probes and the other as a reference FET without oligonucleotide probes. B) Schematic diagram showing measurements of the electrical characteristics of a FET. The shift of the threshold voltage (V_T) was determined from the gate voltage–drain current (V_G–I_D) characteristics in a phosphate buffer solution (0.025 M Na_2HPO_4/0.025 M KH_2PO_4, pH 6.86). An Ag/AgCl electrode with saturated KCl solution was used as reference electrode.

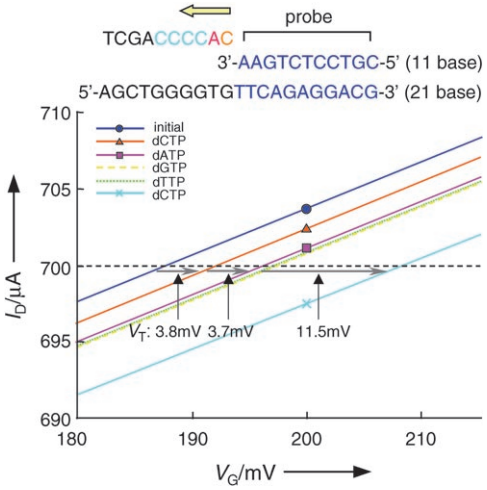

Figure 3. V_T shifts after a single-base extension reaction at the gate surface. The threshold voltages were shifted in the positive direction because of the intrinsic negative charges of deoxynucleotides. The amount of the V_T shift was determined at a constant drain current of 700 μA.

(Table 1[10]) were used to demonstrate the principle of DNA sequencing based on the FET. We paid special attention to the buffer concentration to be used for measuring the change in charge density at the gate surface. The change in potential induced by adsorption of proteins at the gate surface was reported to be dependent on the electrolyte concentration.[11] It is therefore important to optimize the Debye length at the gate insulator/solution interface. Herein, a 0.025 M phosphate buffer solution was used for measuring the change in charge density at the gate surface, whereas a conventional reaction mixture was used for the single-base extension reaction.

Table 1: Base sequences for oligonucleotide probes and targets for the −122 and R353Q regions of factor VII gene and the C282Y regions of hereditary hemochromatosis gene.[10]

Locus	Function	Sequence
R353Q	R353Q wild-type	
	probe	5′-amino group-CCACTACCG-3′ (9mer)
	target	5′-ACGTGCCCCGGTAGTGG-3′ (17mer)
−122	−122 wild-type	
	probe	5′-amino group-CGTCCTCTGAA-3′ (11mer)
	target	5′-AGCTGGGGTGTTCAGAGGACG-3′ (21mer)
C282Y	C282Y wild-type	
	probe	5′-amino group-AGATATACGTG-3′ (11mer)
	target	5′-CTCCACCTGGCACGTATATCT-3′ (21mer)

The 11-base oligonucleotide probes were immobilized on the gate surface and hybridized with the 21-base target DNA for the base sequence of −122 (Table 1[10]). The shifts of the V_G–I_D characteristics, that is, the V_T shifts, were measured after incorporation of deoxynucleotides (Figure 3). When the FET was soaked in a DNA polymerase buffer solution containing dCTP, the V_T shifted in the positive direction by 3.8 mV after single-base extension. Next, the FET was soaked in a DNA polymerase buffer solution containing dATP, and the V_T value shifted further in the positive direction by 3.7 mV, because dATP was incorporated into the probe–target duplex on the FET. When the measurements of the V_T shifts after the single-base extension reaction were performed 15 times, the average V_T shift was 3.2 mV with a standard deviation of 1.1 mV.

When the FET was introduced into a buffer solution containing dGTP and dTTP, the V_T value remained nearly constant because the deoxynucleotides were not incorporated into the noncomplementary base sequence. In this case, the average V_T shift was 0.03 mV with a standard deviation of 0.67 mV. Thus, the V_T shifts based on single-base extension were large enough to be detected and had a sufficient signal-to-noise ratio. Moreover, the V_T value shifted in the positive direction by 11.5 mV when the FET was soaked again in the buffer solution containing dCTP. This is because four dCTP molecules with negative charges were incorporated into the probe–target duplex on the gate surface.

To evaluate the FETs in combination with single-base extension for DNA sequencing, we prepared four kinds of buffer solutions containing both DNA polymerase and either dCTP, dATP, dGTP, or dTTP. The FETs hybridized with target DNA were immersed in these buffer solutions for the single-base extension reaction, and the shift of the V_T value was measured in a phosphate buffer solution (0.025 M) after washing the FETs. The cycle of single-base extension and measurement of the V_T was repeated iteratively to determine the base sequence of the target DNA. When the base sequence of the R353Q region of the factor VII gene was used as target DNA, the V_T shifted in the positive direction only after single-base extension with the specific deoxynucleotides that were complementary to the base sequence of the target DNA (Figure 4 A). The change in V_T for three-base extension, GGG, was 6.9 mV, which was larger than that for one-base extension but was not three times that expected

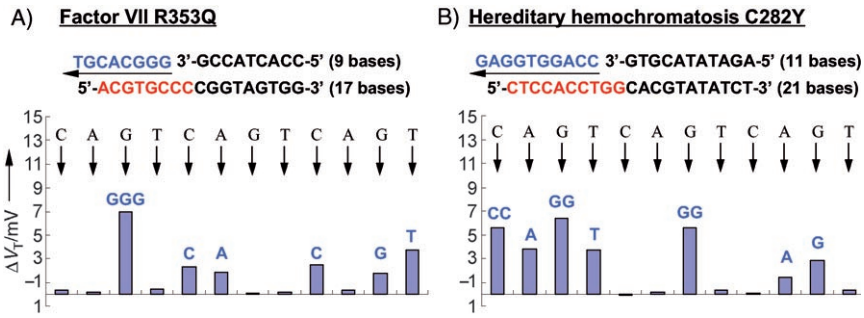

Figure 4. DNA sequencing by the method presented herein. DNA polymerase extends the immobilized oligonucleotide probes with each deoxynucleotide in a template-dependent manner. The base sequences of the R353Q region of the factor VII gene (A) and the C282Y region of the hereditary hemochromatosis gene (B) were successfully determined.

from the number of intrinsic charges. Although the linear relationship between the base length synthesized by the extension reaction and the V_T shift was obtained in the range from 0 to 30 bases,[12] it is important to detect single-base extension quantitatively to reduce the base-call error, especially for continuous sequences of the same base. The density and orientation of the immobilized oligonucleotide probes have to be controlled during a series of extension reactions at 72 °C. Further improvement of the precision of the base call is also expected by automation of the extension reaction and V_T measurements.

The C282Y region of the hereditary hemochromatosis gene was used as another example of DNA sequencing with FETs and single-base extension (Figure 4B). The positive V_T shifts could be detected in accordance with the base sequence of the target DNA. In this case, the average V_T shift for two-base incorporation was 5.8 mV with a standard deviation of 0.4 mV, whereas the average V_T shift for single-base extension was 3.2 mV, as described previously. The V_T shift for two-base extension was approximately twice as big as that for single-base extension. Thus, the results of the iterative extension reaction and detection of the threshold voltage indicated the ability of a direct, simple, and potentially precise DNA sequencing analysis by using the FETs.

The number of bases that can be analyzed by the proposed method is about ten at present. The V_T shift for single-base extension became gradually smaller as the number of bases increased by more than ten bases. One of the reasons for this limitation is the Debye length at the gate insulator/solution interface. Any change in charge density induced outside the Debye length cannot be detected with a FET. The lateral extension reaction in which DNA probes are extended in parallel with the gate surface would be effective for DNA sequencing with long base length. Another reason for the limitation would be the peeling off of the immobilized oligonucleotide probes from the surface of the gate insulator as the temperature stress of the extension reaction at 72 °C is applied repeatedly. A stronger immobilization method for oligonucleotide probes on the Si_3N_4 surface has to be adopted to analyze longer base sequences. The density of the immobilized oligonucleotide probes was calculated by the Graham equation.[9b] As the measured V_T shift was 3.2 mV on average for single-base extensions, the resulting probe density at the gate surface was 7.3×10^{11} cm^{-2}. This value is similar to the reported values for field-effect devices.[13]

The above results demonstrate that single-base extension with any deoxynucleotide can be directly transduced into an electrical signal by the FETs, and that DNA sequencing can be achieved based on the intrinsic charges of DNA molecules without any labeling material. It is possible to integrate multiple FETs and signal-processing circuits in a single chip by using advanced semiconductor technology. Simultaneous analyses of various base sequences that include SNPs can be realized with the FETs. As the output of the FET is an electrical signal, it is easy to standardize the results obtained with the FETs as compared to analyses based on fluorescence detection. Therefore, the platform based on FETs is suitable for a miniaturized and arrayed system for SNP genotyping, as well as DNA sequencing in clinical research and diagnostics.

Experimental Section

Insulated-gate FETs were fabricated by standard integrated-circuit technology except for deposition of the gate electrode. The gate structure and the fabrication process for the FETs are described in detail elsewhere.[14] A temperature sensor and 16 FETs were integrated in a 5×5-mm^2 chip (Figure 2A). We used an n-channel depletion-mode FET with a double layer of Si_3N_4/SiO_2 as the gate insulator on which oligonucleotide probes were immobilized. The thicknesses of the Si_3N_4 and SiO_2 layers were 140 and 35 nm, respectively. The channel width W and the channel length L were designed to be 2400 and 5 μm, respectively, and as a result, the ratio W/L was 480:1. The fabricated FET chip was mounted on a flexible polyimide film with patterned copper electrodes and wire-bonded. The FET chip was encapsulated with an epoxy resin (ZC-203, Nippon Pelnox) except for the gate areas. The typical drifts of these FET devices were about 0.1 mV h^{-1}. Herein, both the fabricated FETs and commercial ion-selective FETs (ISFETs, BAS Inc.) were used. The FETs were immersed in a phosphate buffer solution (0.025 M Na_2HPO_4/0.025 M KH_2PO_4, pH 6.86, Wako) with an Ag/AgCl reference electrode with saturated KCl solution (Figure 2B). The electrical characteristics of the FETs, such as the V_G–I_D characteristics, were measured in a phosphate buffer solution of pH 6.86 at room temperature by using a semiconductor parameter analyzer (4155C, Agilent). The V_T shift was determined after each single-base extension reaction. It was defined as the difference in the V_G–I_D characteristics at a constant drain current of 700 μA.

Oligonucleotides were synthesized by the phosphoramidite method and purified by HPLC (Espec). The 5′-end of the synthesized oligonucleotide probe was modified with an amino group for attachment to the Si_3N_4 surface. The base sequences of the factor VII gene, which include two SNP sites, −122 (21 bases) and R353Q (17 bases), and those of the hereditary hemochromatosis gene, which include C282Y (21 bases) (Table 1[10]), were used as model samples. The base lengths of the oligonucleotide probes were 9 or 11 bases.

The surface of the Si_3N_4 layer was cleaned with 1 M NaOH for 1 h at room temperature and silanized in toluene (Sigma-Aldrich) containing (3-aminopropyl)triethoxysilane (2 wt %, Sigma-Aldrich).

The aminosilanized Si_3N_4 surface was rinsed in toluene and dried in vacuo at 110 °C for 0.5 h. Reactive amino groups were then introduced at the Si_3N_4 surface.

Oligonucleotide probes were immobilized on the modified Si_3N_4 surface by using glutaraldehyde as a bifunctional cross-linking agent. The aminosilanized Si_3N_4 surface was soaked in a glutaric dialdehyde (25 wt %, Sigma-Aldrich) solution with sodium cyanoborohydride (0.5 g per 50 mL, Sigma-Aldrich) for 4 h at room temperature, followed by rinsing in deionized water and drying in vacuo at room temperature for 1 h. Oligonucleotide probes were dissolved in Tris-Cl/EDTA (TE) buffer (pH 8.0, Nippon Gene) at a concentration of 100 μM. To couple the amino-modified oligonucleotides with the glutaraldehyde-treated Si_3N_4 surface, the FET chip was kept at 50 °C in the oligonucleotide solution with sodium cyanoborohydride (0.5 g per 50 mL) overnight to complete the coupling reaction. The FET chip was then soaked in a phosphate buffer solution (0.04 M Na_2HPO_4/0.03 M KH_2PO_4, pH 7.0, Wako) with glycine (1M, Wako) at 50 °C for 1 h to block any remaining glutaraldehyde groups. The FET chip was washed with phosphate buffer solution (pH 7.0) and deionized water, and dried in vacuo at room temperature for 1 h. The oligonucleotide probes were confirmed to be immobilized at the surface of Si_3N_4 by time-of-flight secondary-ion mass spectrometric (TOF-SIMS) analysis.[8b] The surface-modified FET chip was then ready for use in DNA sequencing studies.

The oligonucleotide probes immobilized on the FET chip were hybridized with target DNA. This DNA was prepared by dissolving target oligonucleotides in a hybridization buffer solution, which was composed of 4 × SSC + 0.1 % SDS (Invitrogen, SSC = sodium chloride/sodium citrate), at a concentration of 100 μM. The FET with the oligonucleotide probe was kept in the hybridization buffer solution containing the target oligonucleotide for 12 h at room temperature. After hybridization, the FET was washed with 1 × SSC + 0.03 % SDS, 0.2 × SSC, 0.05 × SSC, and deionized water at room temperature to remove nonhybridized oligonucleotides.

A thermostable DNA polymerase was used for DNA sequencing based on the single-base extension reaction. The reaction mixture contained KCl (50 mM), Tris-HCl (20 mM, pH 8.4), $MgCl_2$ (3 mM), *Taq* DNA polymerase (0.1 U μL^{-1}, Invitrogen), and deoxynucleotide (5 mM of either dCTP, dATP, dGTP, or dTTP; Invitrogen). After hybridization with the target oligonucleotide, the DNA polymerase-assisted synthesis with all four deoxynucleotides was carried out at the gate surface of the FET at 72 °C for 10 min. After incorporation of each deoxynucleotide, the FET was washed with deionized water and dried in vacuo at room temperature.

Received: September 6, 2005
Revised: January 6, 2006
Published online: February 28, 2006

Keywords: DNA · molecular electronics · polymorphism · semiconductors · sequence determination

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