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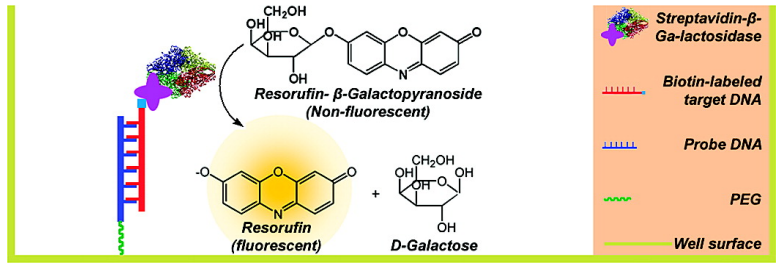
J. Am. Chem. Soc., **2008**, 130 (38), 12622-12623 • DOI: 10.1021/ja8053018 • Publication Date (Web): 03 September 2008



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Published on Web 09/03/2008

Detection of Single-Molecule DNA Hybridization Using Enzymatic Amplification in an Array of Femtoliter-Sized Reaction Vessels

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In this paper, we describe a highly sensitive single-molecule readout scheme to detect biotinylated DNA targets utilizing enzymatic signal amplification in an array of femtoliter-sized reaction vessels. Sensitive DNA detection is extremely important in clinical diagnostics, gene therapy, and a variety of other biomedical applications. Detecting and quantifying trace amounts of specific DNA sequences have relied heavily upon both target amplification and various signal amplification methods.[1–5] Currently, the polymerase chain reaction (PCR) is the most widely used target amplification method owing to its sequence specificity and high amplification yield.[1] In signal amplification, several types of nanomaterials such as quantum dots, silica, and metal nanoparticles, have been employed for bioanalysis.[2–4] Among them, a novel assay based on gold nanoparticle-promoted reduction of silver was reported to detect target DNA.[4] The signal was significantly amplified using this method. The silver reduction process, however, resulted in reduced reproducibility. Alternative methods for trace DNA detection use total internal reflection fluorescence microscopy and reduced detection volumes.[6]

During the past few decades, micro- and nanotechnologies have enabled the generation of densely packed array structures with micrometer-sized elements. These structures allow for small volume isolation,[7] high-throughput, and single molecule PCR amplification,[8] as well as single molecule interrogation.[9] Our previous efforts to develop a proof of concept binding assay for single molecule β-galactosidase detection has been reported. This platform used an array of femtoliter-sized reaction vessels in combination with enzymatic signal generation.[10] Recently, this technology was used to observe enzyme catalytic activity at the single molecule level.[11,12] The ease of performing this assay, along with the simple binary readout, should facilitate the development of this technology for highly sensitive detection of specific biomolecules of interest.

Herein, we demonstrate the further application of this femtoliter-sized reaction vessel array technology for highly sensitive DNA analysis with an experimental limit of 1 fM. To validate the utility of this technology in our study, we employed a fiber optic array to create thousands of femtoliter-sized reaction wells, each specifically functionalized with oligonucleotide probes capable of capturing biotinylated target DNA. After hybridization, the fiber was incubated with streptavidin-labeled enzyme solution. The bound single enzyme molecules were confined to individual reaction vessels containing excess fluorogenic substrate and catalyzed the production of a sufficient number of fluorescent product molecules to generate a detectable signal (Figure 1). At low target DNA concentrations with relatively short incubation times, only a small percentage of the capture sites bind target DNA, enabling a binary readout of target concentration from the high-density fiber array.

The starting point for the array of reaction vessels is a fiber optic bundle comprising ~50000 individual 4.5 μm diameter silica fibers.[10] Both ends of the optical fiber bundle were polished, and the distal end was chemically etched to form reaction vessels. The

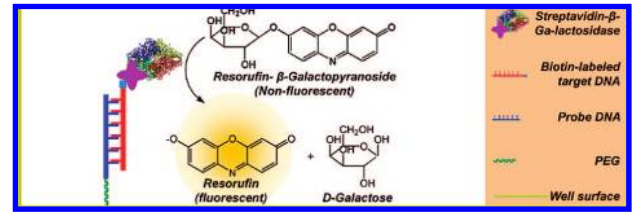

Figure 1. Schematic illustration of DNA-sensing technology performed in femtoliter-sized reaction wells of a fiber array employing enzymatic amplification for signal generation.

cladding around each fiber core is doped with germania, which etches at a slower rate. The vessel volume can be precisely controlled by adjusting the etch time and etchant concentration. The optical fibers used in the present work were etched to a depth of approximately 2.9 μm, yielding an array of 46 fL vessels. The optical fiber bundle is an advantageous platform for bioanalysis, in that the individual fibers in contact with each well can be used to carry both excitation and emission light via total internal reflection to and from the wells, enabling remote interrogation of the well contents. An array of optical fibers also provides the capability for simultaneous excitation of molecules in adjacent vessels without signal "cross-talk" between fibers.[13]

The single stranded DNA probes are covalently attached to the surface of the reaction chambers. This attachment is accomplished by modifying the fiber array with a linker, (3-aminopropyl)triethoxysilane (3-APTES), followed by gentle polishing to remove the amino-silanized layer from the cladding surface. A second linker, homobifunctional PEG (NHS-PEG-COOH), was subsequently allowed to react with the amine group of 3-APTES. The PEG-modified surface, known to be biocompatible and resistant to protein adsorption,[14] was essential as it efficiently suppressed nonspecific binding and enabled the reliable detection of low abundance targets. The carboxyl-terminated PEG linker was then allowed to react with amine-modified single stranded probe DNA (NH_2-DNA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC). This reaction immobilized the DNA probes onto the well surfaces. To test the effectiveness of this surface modification procedure, probe DNA labeled with Cy3 (NH_2-DNA-Cy3) was used, instead of unlabeled NH_2-DNA, with and without EDAC. Fluorescence was observed on the surface only when NH_2-DNA-Cy3 was immobilized with EDAC. Without EDAC, dark wells were observed that were barely distinguishable from those in the background image, indicating the highly efficient blocking by PEG.[15]

After array functionalization, the probe DNA-modified fiber bundles were incubated for 2 h at room temperature in 50 μL of phosphate buffered saline (PBS) containing different concentrations of biotinylated target DNA. To label the DNA with an enzyme, the arrays were then washed repeatedly with TE buffer and PBS

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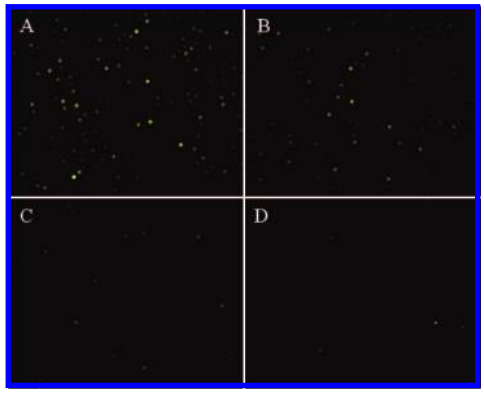

Figure 2. A portion of a probe-modified fiber array, following incubation with complementary target DNA concentrations (A) 100, (B) 10, and (C) 1fM and (D) a noncomplementary control DNA concentration 1 nM. A representative number of active versus inactive reaction wells is shown (pseudocolor added using IPlab software).

with 20% formamide (v/v) to remove unbound targets, followed by a 5 min incubation with streptavidin-β-galactosidase conjugate (SβG). The arrays were then washed three times in PBS with 1% Tween 20 (v/v), and then mounted on an upright fluorescence microscope system equipped with a mechanical platform. A solution of resorufin-β-D-galactopyranoside (RDG), a fluorogenc substrate of β-galactosidase, was added to the distal end of the fiber bundle containing the reaction wells, and the wells were subsequently sealed with a silicone elastomer gasket. This gasket was sandwiched between a microscope slide and the fiber array by means of a mechanical platform located beneath the microscope stage. Uniform pressure applied to the gasket material across the entire bundle sealed and isolated each reaction chamber, enabling interrogation of enzyme activity in individual wells.

At a very low concentration of target DNA, the ratio of the number of DNA molecules to the number of wells assumes a Poisson distribution, where either a single DNA molecule or no DNA binds to each well. When a single biotinylated DNA molecule is hybridized to the immobilized probes in a well, it captures a single SβG enzyme molecule. Each SβG molecule can hydrolyze thousands of RDGs to fluorescent resorufin molecules thereby generating a detectable fluorescence signal in each sealed well, as shown in Figure 2. The variation in intensity among active wells is most likely a result of enzyme-to-enzyme variation in catalytic activity, combined with surface effects that may modulate the relative activities of different enzyme molecules on the basis of their orientation on the reaction well surface.[16,17] As seen in Figure 2A−C, the number of active wells decreased as the concentration of target DNA decreased from 100 to 1 fM. To verify that the binding of SβG was due to the target DNA and not due to nonspecific binding to the glass, a control experiment was performed using 1 nM biotinylated noncDNA, as shown in Figure 2D. The control experiment generated a negligible active-well percentage (less than 0.62% versus 4.52% for 1 fM desired cDNA). Furthermore, statistical analysis was carried out to explore the correlation between the numbers of active wells that captured a target DNA and the concentration of target DNA. In Figure 3, a linear relationship was obtained in the log−log plot of the concentration of target DNA versus the percentage of active wells. This response

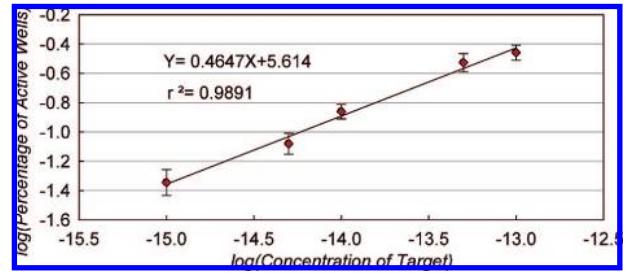

Figure 3. Log−log plot of the target DNA concentration with the resulting percentage of active reaction wells.

is due to the hybridization not yet being at equilibrium. These results show that this binary readout detection method could be reliably employed for highly sensitive DNA detection down to 1 fM, which is comparable to other highly sensitive approaches.[3,4,7,18–21]

In conclusion, we have developed a highly sensitive single molecule DNA assay platform with a 1fM experimental detection limit using enzymatic amplification in an array of femtoliter-sized reaction wells. This simple binary readout-based scheme is easy to perform and exhibits a high signal-to-noise ratio in the presence of trace amounts of DNA target. Furthermore, it also should be possible to extend this technology to protein detection by modifying the reaction wells with specific capture antibodies. We expect this assay to be useful in a number of biomedical applications where accurate and highly sensitive target analysis is critical.

Acknowledgment. We thank Quanterix Corporation for their financial support of this work.

Supporting Information Available: Related instrumentation and experimental procedures, results of surface modification testify. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA8053018