Single-Molecule Detection of Specific Nucleic Acid Sequences

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Introduction

The detection of specific nucleic-acid sequences is of fundamental importance in the fields of genetic and medical research, clinical chemistry, and forensic science, among others. The most common method for the identification of specific DNA sequences is the Southern blot. In this procedure, the DNA sample is cleaved with a restriction enzyme, size-separated by gel electrophoresis, and transferred from the gel to a nitrocellulose filter. Detection is then accomplished by adding a hybridization probe. Despite its popularity, Southern blotting suffers from some limitations, mainly because it involves a series of manually intensive procedures that cannot be run unattended and cannot be readily automated; casting gels, applying samples, and running and subsequently staining the gels are all time-consuming tasks that are susceptible to poor quantitative accuracy and poor reproducibility. In most cases, in order to improve sensitivity, a radioisotope must be incorporated into the probe, which brings up a set of safety and environmental concerns.

The development of various techniques for enzymatic amplification of the target sequence before analysis alleviates the sensitivity problem. The polymerase chain reaction (PCR), for example, selectively increases the concentration of the target sequence relative to unrelated sequences, thus enhancing both the specificity and sensitivity of the assay. Amplification methods, however, may introduce ambiguities resulting from contamination, from variability in amplification efficiency and from other mechanisms not fully understood.[1–6]

The need, therefore, exists for economical, automatable methods for the analysis of small amounts of unamplified DNA in the high-throughput analytical laboratory. Recent developments in laser-based detection of fluorescent molecules have made possible the implementation of single-molecule techniques for biochemical analysis. The basis of our approach is to monitor for the presence of a specific nucleic-acid sequence of bacterial, human, plant, or other origin. The nucleic-acid sequence may be a DNA or RNA sequence and may be characteristic of a specific taxonomic group, a specific physiological function, or a specific genetic trait. These techniques have been developed with the following desired properties:

- high sensitivity: detecting a single target-molecule, allowing the analyst to circumvent the use of enzymatic amplification reactions;
- high specificity: detecting a short sequence in the presence of a large genomic background;
- homogeneous assay format: permitting the use of simple reaction protocols, which are amenable to automation; and
- high throughput: obtaining the desired statistical confidence of detection in a short period of time.

Instrumentation for Single-Molecule Detection in Flow Capillaries

The preferred experimental scheme for single-molecule detection of DNA fragments tagged with fluorescent probes for high-throughput analysis is the photon-burst detection with time-gating electronics, because of the high detection throughput and excellent signal-to-noise ratios that are possible.[7–12] The Photon-Burst Detection in Flowing Solutions method consists of pumping a dilute solution of the sample under investigation through a small capillary or flow cell. The concentration of fluorophore (a molecule that fluoresces when excited by light) in this kind of experiment is typically so low that the probability that two molecules would occupy the same volume at a given instant is negligible.

A laser beam is tightly focused through the flow cell. As the molecules travel through the path of the laser beam, they are excited; they subsequently decay by the emission of their characteristic fluorescence radiation (see Figure 1). Flow-rate adjustments limit the residence time of a molecule in the laser beam to the order of a few milliseconds. Because the fluorescence lifetime of most common fluorophores is of the order of a few nanoseconds, a single molecule undergoes thousands of excitation-emission cycles during its travel through the laser beam, which produces a fluorescence photon burst. This fluorescence is collected at right angles to the excitation beam by high numerical aperture (N.A.) optics. A spatial filter at the image plane rejects out-of-focus scattering of the laser beam. The excitation light from the laser is blocked from reaching the detector by a bandpass spectral filter with a transmission band that overlaps the molecular fluorescence spectrum. A single-photon avalanche photodiode is usually used as the detector because of its high detection quantum efficiency and fast response time. In spite of the use of spatial and spectral filters, large amounts of incompletely attenuated excitation light, as well as Raman scattering from the solvent, reach the detector. By using a picosecond—or shorter—laser as the excitation source, it is possible to reject the majority of the background scattering. These two scattering emissions occur only during the duration of the laser pulse, whereas fluorescence occurs up to several nanoseconds after the excitation pulse. Therefore, by setting an electronic time-gate window that includes only delayed photons, one can reject the scattered light and retain the majority of the desired fluorescence signal.[7]

Figure 2 shows a schematic representation of our apparatus.

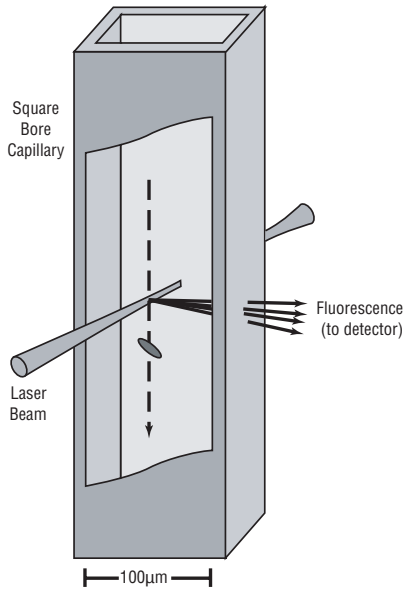

Figure 1. Schematic diagram of the flow cell for single-molecule detection.

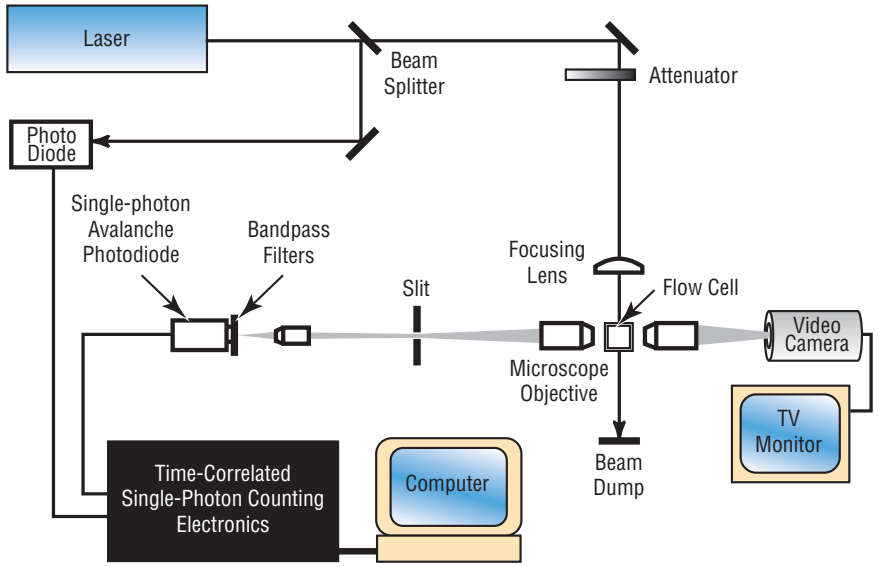

Figure 2. Schematic diagram of the experimental setup for fluorescence photon burst detection of single-molecules in flowing solution.

The excitation source is the frequency-doubled output (532 nm) of a mode-locked Spectra-Physics model 3800 Nd:YAG (neodymium:yttrium-aluminum-garnet) laser that produces 70-ps pulses at 82 MHz repetition rate. The laser output is attenuated to 2–5 mW and focused by a 6× microscope objective into the 0.8 × 0.8 mm ID square cross-section capillary cell to yield a 10-μm spot. A syringe pump is used to pump the sample through the capillary cell at a rate of 200 μL/hr, which translates to an average linear flow velocity of 87 μm/s. As individual molecules move through the laser beam, repeated excitation-emission cycles produce a fluorescence photon burst. The apparatus incorporates two detection channels so that it can detect the photon bursts from two dyes, each with different spectral properties, simultaneously. Fluorescence in each channel is collected by a 40 × 0.85 N.A. microscope objective and spatially filtered by a 0.4 × 0.4 mm square slit, which defines a 10 × 10 μm detection area. Omega (RDF series) 30 nm-bandwidth eight-cavity interference filters then spectrally filter the light. The filter bandpass on each channel is chosen to achieve sufficient overlap with the emission spectrum of the corresponding dye but negligible overlap with the spectrum of the other dye. EG&G single-photon avalanche photodiodes detect the light. Independent time-correlated single-photon counting electronics under computer control analyze each detector output signal. The detection electronics reject Raman and Rayleigh scattering by using a time-gate window set such that only delayed fluorescence photons are detected. Fluorescence data from each channel is usually collected in 1-ms intervals, for a total running time that ranges from 100 to 1000 s, and saved for later analysis.

Figure 3 shows the experimental results for the detection of single rhodamine 6G molecules in a 5-fM aqueous solution. Large-amplitude bursts that correspond to individual molecules are clearly evident. Molecules that pass through the center of the gaussian laser beam yield as many as 150–200 photons, whereas smaller bursts are caused by molecules that pass through the edges of the beam.

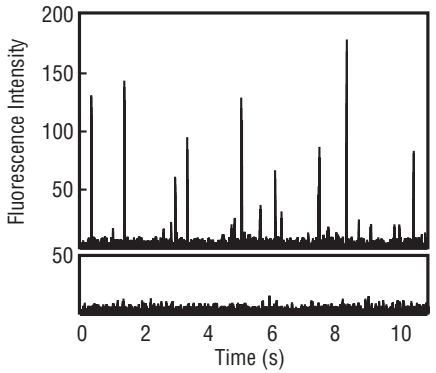

Figure 3. Photon-burst raw data for a 10-fM rhodamine 6G aqueous solution (top) and neat water (bottom). Laser power: 5 mW, flow velocity: 200 μm/s.

Double-Label Assay

The double-label assay detection scheme involves the use of two nucleic-acid probes that are complementary to the DNA target.[13–14] Peptide nucleic acids (PNA) are used instead of DNA probes because PNAs exhibit stronger binding to DNA targets.[15] In PNAs, the entire deoxyribose phosphate backbone has been replaced by a chemically different but structurally homomorphous backbone composed of (2-aminoethyl) glycine units[16-17] (see Figure 4). PNA probes have been found to be very potent DNA mimics, forming Watson-Crick base-paired duplexes with complementary DNA of high specificity and thermal stability.[18] In our experiments, we hybridized two PNA probes, each labeled with a different fluorescent dye, to the sample. If a target molecule that has both probe binding sites is present, then a complex should form between the target and the two probes. The two-channel single-molecule detector then analyzes the sample. Because the probes are bound to the same DNA target fragment, their signals will appear at the same time. Thus, the simultaneous detection of the two probes signifies the presence of a target molecule (see Figure 5). When no target is present, the probes will emit signals that are not coincident in time. Noncoincident signals will result from unhybridized probes, from targets hybridized to only one probe, or from nonspecifically bound probes. Such background signals are readily distinguished from the coincident signals generated by a target molecule as demonstrated in the following experiments.

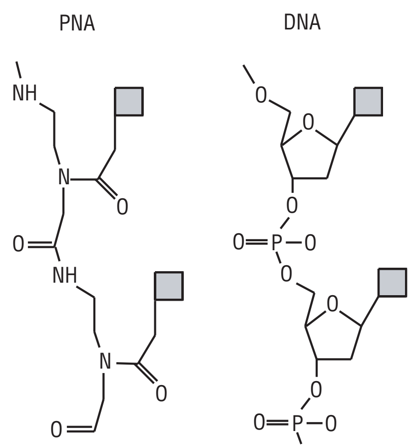

Figure 4. Structure of the peptide nucleic acid analog. The phosphate backbone is replaced by a chain of (2-aminoethyl) glycine units.

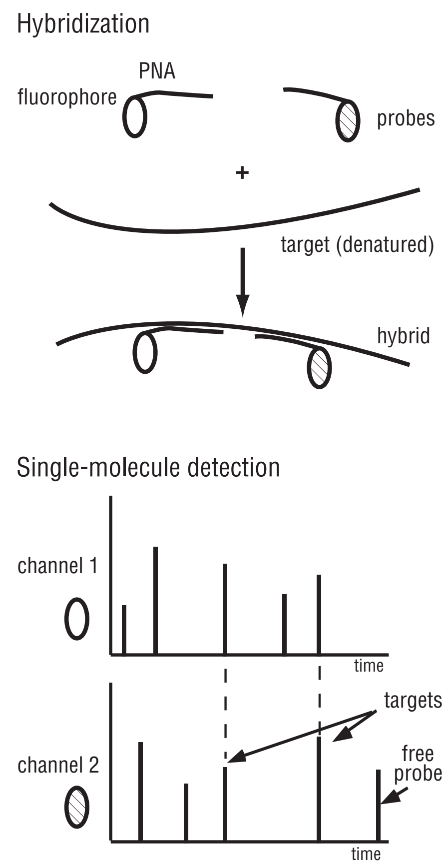

Figure 5. Schematic diagram of the double-label coincidence assay. Simultaneous detection of the two hybridized probes allows the detection of a specific sequence of the target at the single-molecule level of sensitivity.

Detection of lambda DNA in the presence of genomic background

In this case, we used two PNA probes labeled with different fluorescent dyes, rhodamine 6G and Bodipy Texas Red, to identify the lambda DNA target sequence. These probes have sequences that are complementary to target sequences at positions 48406 and 48425 respectively. Single molecules of the probe-target complex are expected to be identified by the coincident detection of both dyes, while unbound probes or single probes bound to randomly occurring complementary sequences should appear as independent signals.[19] We prepared the sample by mixing lambda and salmon DNA in a genomic ratio of 1:1. The salmon DNA provides a background of unrelated sequences to test specificity for the lambda target. The hybridized sample was loaded into the system at a final concentration of 5 fM. Figure 6 shows the raw data obtained (top and middle traces). It is apparent that some signals occurred in only one of the channels (independent signals), while others occurred simultaneously in both channels (coincident signals). Cross-

correlation analysis yields a large peak at zero lag time (see Figure 6, bottom trace). A control experiment, described in the next paragraph, shows that the coincident signals above background are due to the binding of two probe molecules to one complementary target sequence, and not to nonspecific probe-DNA complexes or from PNA probe complexes unrelated to the presence of DNA.

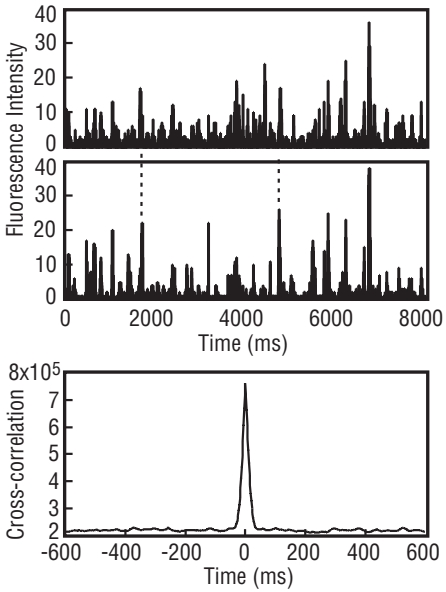

Figure 6. Single-molecule fluorescence photon bursts for intact lambda DNA hybridization mixture samples. Top: rhodamine channel raw data. Middle: Bodipy Texas Red channel raw data. Two representative coincident bursts are indicated by dashed lines. Bottom: cross-correlation analysis.

To see whether the coincident signals of Figure 6 indicate the presence of specific probe-target complexes, we repeated the experiment under identical conditions, except that the lambda target was cut between the two probe binding sites before mixing with the salmon DNA and hybridizing to the probe. Probes should still bind to target sequences as before, but coincident signals should be eliminated because the binding sites would now be on independent target fragments. On the other hand, if the coincident signals were due to nonspecific complexes, cleavage of the target DNA should have no effect on the occurrence of coincident signals. Photon bursts were recorded and analyzed as before (see Figure 7). Cross-correlation analysis revealed no coincident events, which shows that the coincident signals seen with intact lambda DNA are due to specific probe-target complexes. We conclude that coincidence detection of two probes provides the specificity required to identify single-copy target sequences in complex samples.

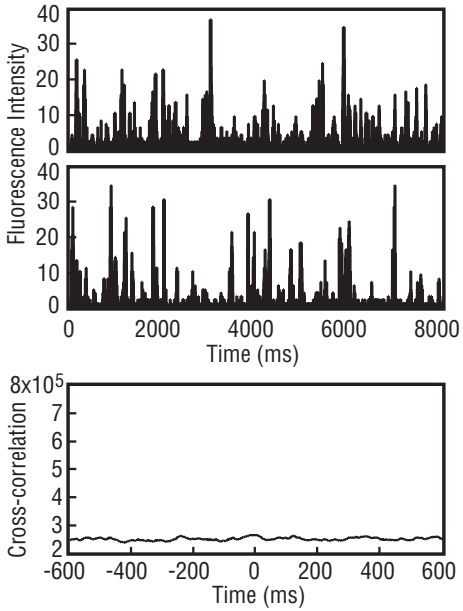

Figure 7. Control experiment for the analysis of lambda DNA in Figure 6. Top: rhodamine channel raw data. Middle: Bodipy Texas Red channel raw data. Bottom: cross-correlation analysis. No coincident signals were found when the target was cleaved between the two probe binding sites.

Detection of a single-copy gene in a transformed maize plant

We obtained the experimental results of Figures 6 and 7 with a synthetic sample comprising a mixture of lambda and salmon DNA. To confirm these results with natural samples, we prepared DNA from a homozygous-transformed maize plant containing one copy of a BT (*Bacillus thuringiensis* toxin) transgene per haploid maize genome (3×10^9 bp). The two probes used in this case (15-mers) hybridize at two positions 620 nucleotides apart in the sequence of the BT gene.[20] Instead of using cleaved DNA for a negative control as in the lambda experiments, here the control is an isogenic plant that lacks the BT gene. Photon bursts were recorded as before. We observed coincident signals only with the BT-positive sample (see Figure 8, top trace) and not with the negative control (Figure 8, bottom trace). This example illustrates that coincidence single-molecule detection can identify a single-copy transgene integrated in a chromosome of a complex genome.

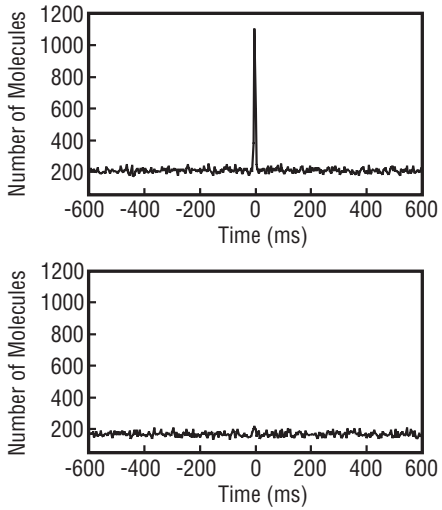

Figure 8. Detection of a single-copy BT transgene in a maize genomic sample. Cross-correlation for the BT-positive (top) and BT-negative (bottom) samples.

Detection of a specific sequence of a pathogen

We have also demonstrated the use of this technique for the detection of *Bacillus anthracis* DNA in solution. *B. anthracis* is a gram-positive endospore-forming bacterium capable of producing fatal infections in both livestock and humans.[21] Virulent strains of *B. anthracis* are encapsulated and cause death in humans and animals by producing various toxins.[22,23] The detection of a specific sequence of *B. anthracis* in the presence of excess amounts of unrelated DNA from salmon sperm, and in the presence of large amounts of genomic DNA from a related bacillus (*B. globigii*), have been accomplished, as described below.

The *B. anthracis* DNA content consists of a 5.7-Mb genome, the 184-kb pXO1 virulent plasmid, and the 95-kb pXO2 capsule plasmid. Our target samples consisted of a 1:1 mixture of the genomic and pXO2 plasmid components at a concentration of either 5 fM or 0.5 fM. In one series of experiments, we added salmon sperm DNA to the target sample at increasingly larger ratios (Table 1), in order to simulate the large amounts of unrelated background DNA usually found in environmental samples. In another case, we added a 100:1 genomic excess of *B. globigii* to a 5-fM *B. anthracis* sample in order to test the sensitivity and specificity of the technique for detecting the pathogen in the presence of another—closely related—bacillus. In all cases, the DNA target samples were denatured by heating at 95°C and then cooled to room temperature. We used two fluorescently labeled, 12-base PNA probes, one tagged with rhodamine 6G (Rho) and the other with Bodipy Texas Red (BTR). These probes have sequences that are complementary to a short region of the capB gene of the pXO2 plasmid, corresponding to nucleotides 475 to 486 and 1082 to 1093, respectively (H-Rho-O-CTGGTACATCTG-$CONH_2$ and H-BTR-O-TGATCCCTCATC-$CONH_2$). The PNA probes were added to the samples at a final concentration of 2 pM and allowed to hybridize to the target for 30 minutes. Immediately afterwards, the samples were loaded into the instrument and single-molecule data was collected as described above.

Table 1. Sample Concentrations Used for the Detection of *B. anthracis* DNA in the Presence of Salmon Sperm DNA

	Expt 1	Expt 2	Expt 3	Expt 4
B. anthracis concentration (fM)	5	5	0.5	0
B. anthracis w/v (pg/ mL)	5	5	0.5	0
Salmon sperm (ng/mL)	0	100	100	100
Mass ratio	—	2×10^4	2×10^5	—
Number of fragments ratio*	—	4×10^4	4×10^5	—

* for an average sheared salmon sperm DNA fragment size of 700 base pairs.

Detection of B. anthracis DNA in the presence of salmon sperm DNA

Table 1 summarizes the various experiments and controls performed in this case. Experiment 1 (see Figure 9a) corresponds to the detection of *B. anthracis* alone at a concentration of 5 fM. The large cross-correlation peak in Figure 9a is an indication of the relative amount of *B. anthracis* DNA present in the sample. Experiments 2 and 3 (see Figures 9b and 9c) correspond to the detection of *B. anthracis* DNA in the presence of large amounts of salmon-sperm background DNA. Experiment 4 (see Figure 9d) was a control where no *B. anthracis* was added and only background DNA was present. No target signal was detected in this case (see Figure 9d).

Detection of B. anthracis DNA in the presence of B. globigii DNA

In this case, we added the complete genome of *B. globigii* to a 5-fM sample of *B. anthracis* DNA at a genomic ratio of 100:1. Figure 10 shows the cross-correlation results (top trace in the graph). The large peak indicates the presence of the target. A control experiment was run under identical conditions, except that *B. anthracis* DNA was not added to the sample (see Figure 10, bottom trace in the graph). No cross-correlation peak was observed in this case, indicating that *B. globigii* does not contribute to the signal.

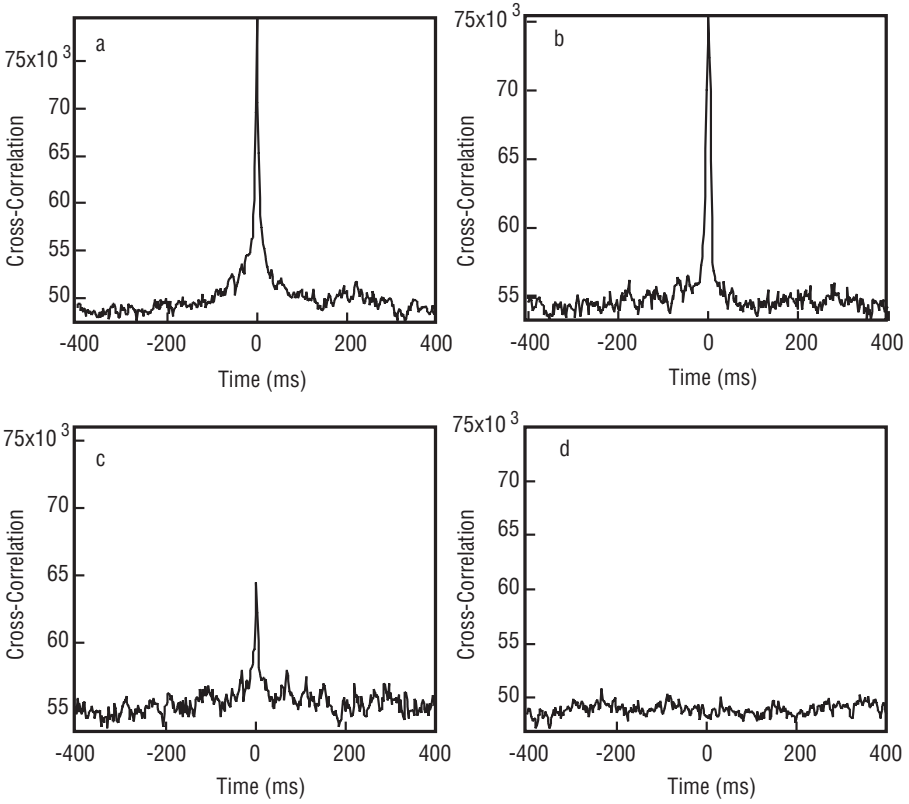

Figure 9. Cross-correlation results for the detection of *Bacillus anthracis* DNA. (a) 5 fM *B. anthracis* alone. (b) 5-fM *B. anthracis* DNA in the presence of 2×10^4 -times excess (by weight) salmon sperm DNA. (c) 0.5-fM *Bacillus anthracis* DNA in the presence of 2×10^5 -times excess (by weight) salmon sperm DNA. (d) Control: Salmon sperm DNA alone.

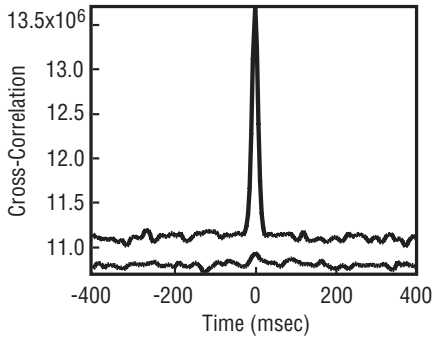

Figure 10. Detection of *Bacillus anthracis* in the presence of *Bacillus globigii*. Top: 5-fM *B. anthracis* DNA and 100× *B. globigii* genomic excess. Bottom: *B. globigii* alone.

PNA Clamp Probes

This method enables the detection of specific nucleic-acid sequences in double-stranded DNA. The detection scheme involves the use of a specially designed homopyrimidine PNA clamp probe that binds to a complementary sequence in a double-stranded DNA target. Homopyrimidine-PNA oligomers form triplex structures with complementary homopurinic sequences and have been found to recognize double-stranded DNA targets by a mechanism that involves displacement of the pyrimidine DNA strand.[24,25] A PNA clamp probe binds to the complementary DNA target strand by forming a very stable local triplex in which one PNA arm is bound by Watson-Crick base-pairing and the other arm is bound by Hoogsten base-pairing to the central purinic strand[26] (see Figure 11). The noncomplementary DNA strand is displaced into a loop structure.[27,28] In addition to the PNA clamp probes, we added an intercalating dye to the sample, which provides a signature for the passage of each fragment through the detection volume. Simultaneous detection of the intercalator fluorescence signal and the labeled probe signifies the presence of the target molecule. When no target is present, the free probe will emit a signal that is not coincident in time with that of the free intercalator. Therefore, the analysis of genomic samples without the need for a denaturing step becomes possible.

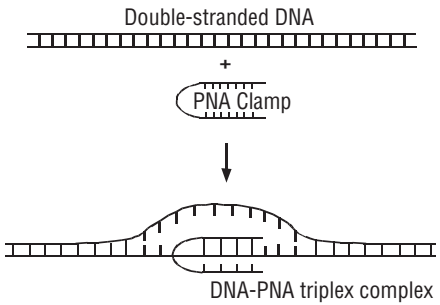

Figure 11. Schematic representation of a PNA-DNA triplex structure. One PNA arm is bound by Watson-Crick base-pairing to the target DNA sequence, and the other PNA arm is bound by Hoogsteen base-pairing to the central purinic strand. The noncomplementary DNA strand is displaced into a free loop structure.

An important property of intercalator dyes is that they bind stoichiometrically to the double-stranded DNA target (*i.e.*, the number of intercalator molecules is proportional to the size of the target).[29] Therefore, after delivering homogeneous excitation to the sample volume, measuring the intensity of the detected photon burst that originates from a target that contains the intercalator allows the determination of the length of the DNA target. The simultaneous use of a sequence-specific probe and an intercalator dye will allow the determination of both the quantity and molecular weight of specific target DNA molecules in complex samples. An additional advantage of this technique arises from the fact that only one probe is used, and, therefore, the hybridization kinetics are faster than the two-probe methods.

Our preliminary experiments have shown the detection of a specific DNA sequence in double-stranded lambda DNA. We hybridized a PNA clamp probe labeled with rhodamine to base position 23892 of lambda DNA. The intercalator dye used was TOTO-3, which has an absorption peak at 642 nm and an emission peak at 660 nm. We used a helium-neon laser, which emits at 633 nm, as the excitation source. Figure 12 shows the experimental results. The top trace shows the raw data for the rhodamine channel, whereas the middle trace shows the raw data for the TOTO-3 channel. A cross-correlation between these two data sets (bottom trace) reveals a peak at zero time that is indicative of the simultaneous detection of the clamp probe and the intercalator dye on the same DNA fragment.

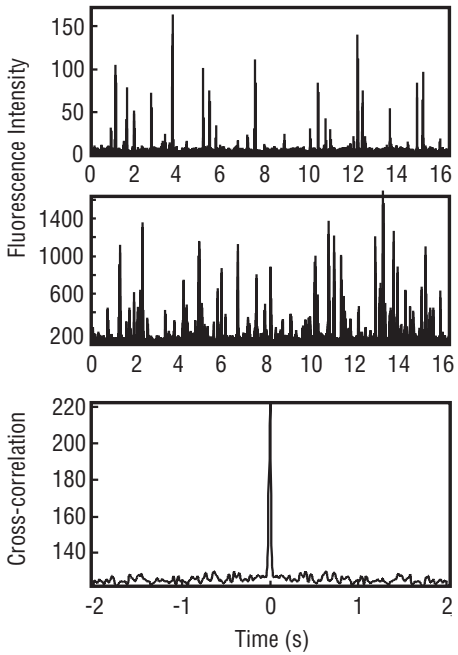

Figure 12. Detection of a specific sequence of double-stranded lambda DNA by the PNA clamp probe method. Top: rhodamine-labeled clamp probe channel. Middle: TOTO-3-stained lambda DNA channel. Bottom: cross-correlation analysis.

Polymerase-Extended Fluorescence Reporters

This method consists of synthesizing a highly fluorescent nucleic-acid reporter molecule, using a sequence of the target as a template. Detection of this highly fluorescent target in our single-molecule detection apparatus signifies the presence of the target being sought. We used a short oligonucleotide primer that is complementary to the target to synthesize the reporter molecule and added suitable polymerase and free nucleotides to the sample. One of these oligonucleotides is—at least partially—labeled with a fluorophore. If the target is present in the sample, the primer binds to it, and the polymerase will incorporate the labeled and unlabeled nucleotides reconstructing the target's complementary sequence (see Figure 13). If the labeled nucleotide concentration is kept below that of the unlabeled nucleotides, most of it will be incorporated into the reporter DNA molecule. Nonetheless, some free labeled nucleotide will remain in the reaction mixture, but fluorescence from the reporter molecule will be much stronger than that of the free nucleotide background over the detection time. Detection of the reporter signifies the presence of the target being sought. The fluorescent signal from the reporter molecule is expected to be much larger than that of the background originating from free labeled nucleotides because the reaction is allowed to proceed until the reporter molecule is hundreds or thousands of bases long.

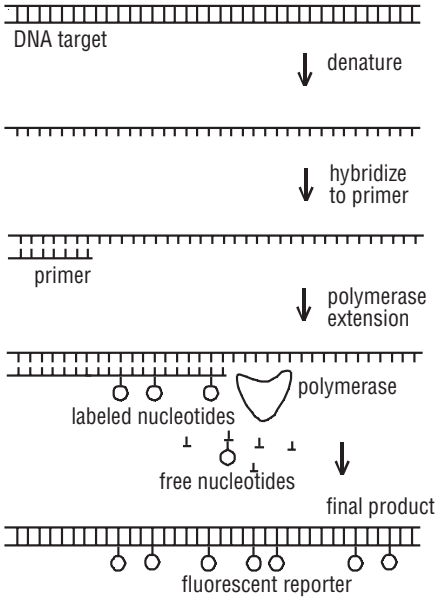

Figure 13. Schematic representation of the sequence-specific synthesis of a fluorescent reporter.

This method has been demonstrated for the detection of pUC19 DNA, (a 2686 base-pair plasmid). Before all experimental procedures, we digested pUC19 DNA with the restriction endonuclease Bgl I, which yields two fragments, 1568 bp and 1118 bp in length, and added a 24-mer primer that anneals to nucleotides 352–375 of pUC19, along with Taq polymerase and a mixture of dATP, dGTP, dCTP, and partially labeled dUTP. As a control, we ran identical experiments, except that pUC19 DNA was substituted with lambda DNA. We used a single-channel, single-molecule detection apparatus as described above to detect fluorescence from the reporter molecule. Figure 14 shows the results for the detection of a specific sequence of the 1568-bp pUC19 fragment at the single-molecule level of sensitivity (top trace). The lambda DNA control yielded negative results (bottom trace).

A possible enhancement to this technique is to simultaneously perform "single-molecule electrophoresis" on the sample.[13] In this method, the electrophoretic mobility of fluorescently labeled molecules (free labeled nucleotides and reporter molecules in this case) can be determined with single-molecule sensitivity. Because single nucleotides exhibit an electrophoretic mobility vastly different to that of nucleic acid targets, interference from free nucleotides is further eliminated. This modification to the method also allows simultaneous determination of the size of the target.

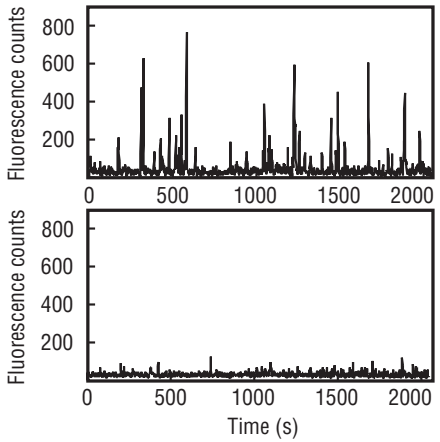

Figure 14. Detection of a specific DNA sequence by the fluorescent reporter method. Top: Raw data for the detection of the 1568-bp pUC19 fragment. Bottom: Raw data for the lambda DNA control.

Conclusions and Outlook

The methods described here promise to combine the advantages of flow-based analytical systems (system automation, speed, reproducibility) with the unsurpassed sensitivity of single-molecule detection. The sensitivity of these methods allows for the direct detection of specific genes without the need for using amplification methods such as PCR and exhibits advantages over current methodologies in terms of sensitivity, specificity, and speed. Also, the high sensitivity of the method means that sample size and reagent use are minimal, which should result in significant cost savings relative to existing analytical methods. Ultimately, assay reliability and low operating costs, combined with high sensitivity, may be the primary advantages of using single-molecule detection methods in the analytical laboratory.

We anticipate that the nonradioactive approaches for the ultrasensitive detection of specific sequences described here will find applications in a wide variety of fields, such as gene identification, gene mapping, medical diagnostics, and biotechnology. When the size of the target is also determined, it will be possible to determine both the quantity and molecular weight of specific target DNA molecules in complex samples, without the need for DNA amplification. The simplicity of the assay chemistry (probe hybridization in solution phase under DNA-denaturing conditions) promises reliability. The high level of target specificity demonstrated in the present experiments suggests that single-molecule detection coupled with single-molecule electrophoresis could be used successfully for many applications in analytical genetics. The inheritance of genes and chromosome segments could be tracked using DNA markers such as restriction fragment length polymorphisms (RFLPs) or tandem repeats. Single-nucleotide differences could be detected by probe hybridization as has been shown for PNA probes.[30] Finally, specific mRNA transcripts could be counted and sized.

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