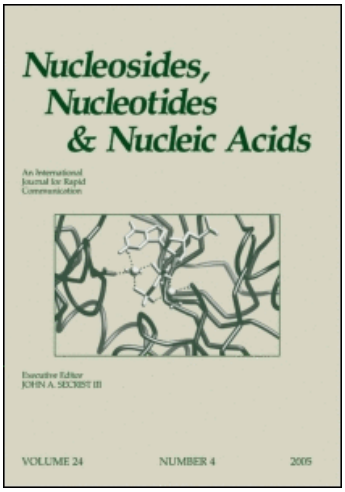

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Peter M. Goodwin[a]; Hong Cai[a]; James H. Jett[a]; Susan L. Ishaug-Riley[a]; Nicholas P. Machara[a]; David J. Semin[a]; Alan Van Orden[a]; Richard A. Keller[a]

[a] Los Alamos National Laboratory, Los Alamos, NM

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APPLICATION OF SINGLE MOLECULE DETECTION TO DNA SEQUENCING

Peter M. Goodwin, Hong Cai, James H. Jett,
Susan L. Ishaug-Riley, Nicholas P. Machara, David J. Semin,
Alan Van Orden, and Richard A. Keller*

Los Alamos National Laboratory, Los Alamos, NM 87545

ABSTRACT : A flow cytometric, single molecule approach to DNA sequencing is described. A single, fluorescently labeled DNA fragment is suspended in a flow stream. An exonuclease is added to sequentially cleave the end base into the flow stream where it is detected and identified by laser-induced fluorescence.

INTRODUCTION

We, and others, have developed the ability to detect single fluorescent molecules crossing a focused laser beam.[1] Assays at the single molecule level yield information that would be difficult or impossible to obtain by bulk measurements.[1] Here, we describe our approach to single molecule detection and discuss its application to DNA sequencing.

SINGLE MOLECULE DETECTION

Our approach to single molecule detection is based upon flow cytometry.[2] Samples are introduced from a small capillary inserted into the center of a square-bore flow cell. Hydrodynamic focusing[2] of the sample by a sheath fluid flowing around the capillary is used to form sample streams ~ 10 μm in diameter that pass through the center of a focused laser beam. Fluorescence is detected at right angles to the sample flow and the excitation beam with a high numerical aperture microscope objective; spatially filtered with a narrow slit located at the image plane; spectrally filtered with the appropriate optical filters to reduce background from Raman and Rayleigh scattered light; and impinged onto the active surface of a photomultiplier tube, microchannel plate, or silicon avalanche photodiode. The detection volume, defined by the image of the spatial filter on the sample stream and the spatial extent of the excitation laser beam in the center of the flow cell, is approximately one picoliter. A mode-locked laser provides ~ 200 picosecond laser pulses and time-gated detection is used to further discriminate against Raman and Rayleigh scattered light.[3] As a

single molecule passes through the laser beam, it is repeatedly cycled between the ground state and the first excited singlet state, emitting a fluorescence photon on most cycles (determined by the fluorescence quantum yield). The resulting signal is a burst of photoelectrons (PE) with a duration up to the transit time of the molecule across the laser beam (typically 1 ms in our apparatus). With careful alignment, the sample stream passes through the center of the detection volume and results in a uniform burst size from each molecule. A detailed description of our apparatus is provided in Reference 4 and 5.

Fluorescent impurities in buffer and enzyme solutions contribute a background that can make single molecule detection difficult. We have shown that photobleaching of buffer and solvent solutions results in a significant reduction of background fluorescence.[6] The sheath fluid, containing buffer and enzyme, is passed through a long capillary tube prior to entering the flow cell. A few hundred mW of the excitation laser beam is split off and passed down the center of the capillary. In this manner, fluorescence from impurities in the sheath stream can be reduced by over an order of magnitude without reducing the enzyme activity.[6]

Signals from single molecules of tetramethyl rhodamine isothiocyanate (TRITC) passing through the laser beam are shown in Fig. 1 (top).[5] A blank containing only buffer is shown in Fig. 1 (bottom). Bursts from individual TRITC molecules are distinguished easily from bursts and background associated with fluorescent impurities in the solvent. The resulting burst size distribution is shown in Fig. 2. Setting a threshold at a burst size of 20 PE results in the detection of > 90% of the molecules eluted from the capillary with few false positives from background. Fluorescence bursts from single molecules of TRITC labeled cytidine, dissolved in Tris-HCl buffer, pH8, are shown in Fig. 3.

DNA SEQUENCING

We are developing an approach to DNA sequencing that has the potential to sequence long fragments of DNA at a rate of 100 to 1000 bp per second.[7-9] Even more important than the projected sequencing rate, is the projected ability to sequence long fragments. We expect to sequence an entire 40-kbp fragment in a single run, thereby reducing greatly the need for sequencing overlaping regions characteristic of the conventional sequencing process. In contrast to gel electrophoresis, our approach is not limited to 1000 bp fragments or by current cloning bottlenecks.

Our flow cytometric approach to DNA sequencing relies on: fluorescent labeling of a single strand of DNA with base-specific fluorescent tags; anchoring the labeled DNA in the center of our flow chamber; digesting the labeled DNA with an exonuclease that sequentially cleaves the end nucleotide; detection and identification of individual,

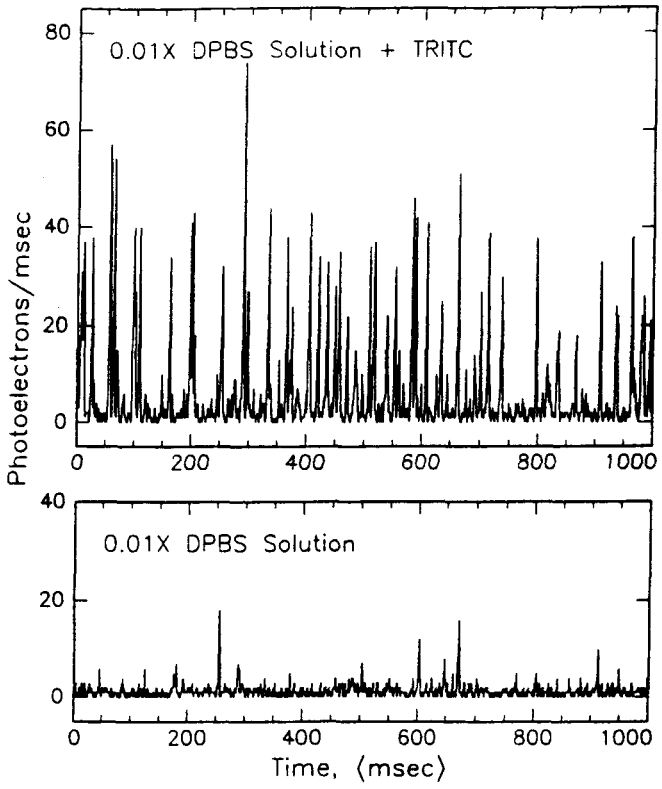

FIG. 1. Detection of Single TRITC Molecules in 0.01X Dulbecco's Phosphate-Buffered Saline (DPBS) Solution.

fluorescently tagged DNA bases by laser-induced fluorescence as they pass through the focused laser beam. The status of each of these steps is discussed below.

Fluorescent labeling of DNA

M13 (circular DNA containing ~ 7000 bp) has been replicated with two normal nucleotides (dATP, dGTP), fluorescinated dCTP, and fluorescinated dUTP. The replication proceeded well and fully replicated M13 DNA was obtained. Fidelity assays indicated that the rate of misincorporation was less than 10^{-5} - the same as with normal nucleotides.[8]

Phagemid was replicated with two normal nucleotides, fluorescinated dCTP and dUTP, or fluorescinated dUTP and dATP. DNA products at least 7000 nucleotides long were obtained[10] A 3000 bp segment of phagemid is 70% A, T; therefore, the replicated DNA has a region where 70% of the bases are tagged when using fluorescinated dUTP and

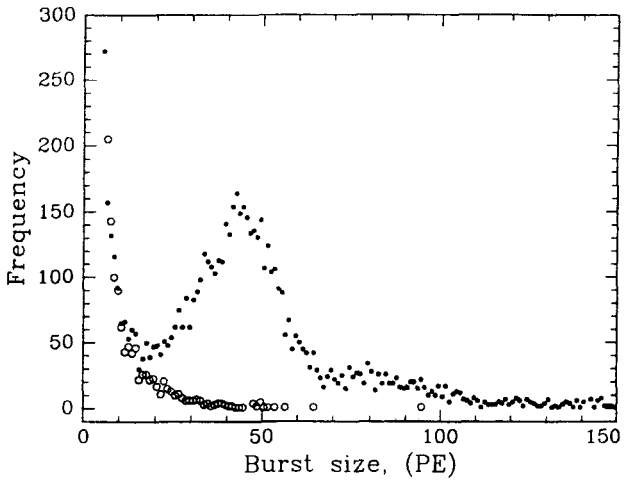

FIG. 2. Efficient detection of single TRITC molecules. Closed circles, sample on; open circles, sample off. Capillary ID < 1μm; excitation laser 20 mW at 554 nm; e^{-2} beam diameter 15 μm; transit time 2 ms.

dATP. Replication out to several thousand bp was observed with Fluorescein-dUTP, Rhodamine-dCTP, Rhodamine-dATP and dGTP.[10] No problems were observed with solubility and basepairing.

Attaching a single, labeled fragment to a microsphere

Biotinylated λ DNA was incubated with 2.8 μm diameter, avidinated, latex beads and stained with the intercalating dye, TOTO.[9] The number of DNA fragments attached to a bead was found to follow a Poisson distribution; determined by counting the fluorescent spots under a microscope. At the optimum ratio of beads to DNA fragments, ~ 70% of the beads had no DNA, ~ 30 % had one DNA fragment, and less than 10 % had more than one fragment. A similar procedure was used to attach fluorescinated M13 to 10 μm beads in the experiments described below.

Mounting the microsphere containing the DNA fragment in the flow cell

A 240 μm o.d., 100 μm i.d. capillary was drawn to a ~ 2 μm tip. A 10 μm bead containing DNA was then attached to the end of the capillary by applying mild suction and the bead and capillary were inserted into the center of the flow cell with the bead ~ 50 μm upstream from the excitation laser beam.

We are exploring optical trapping as an alternative to the micropipette for holding the bead in the flow cell.[11,12] Microspheres are trapped from a flowing steam and held in

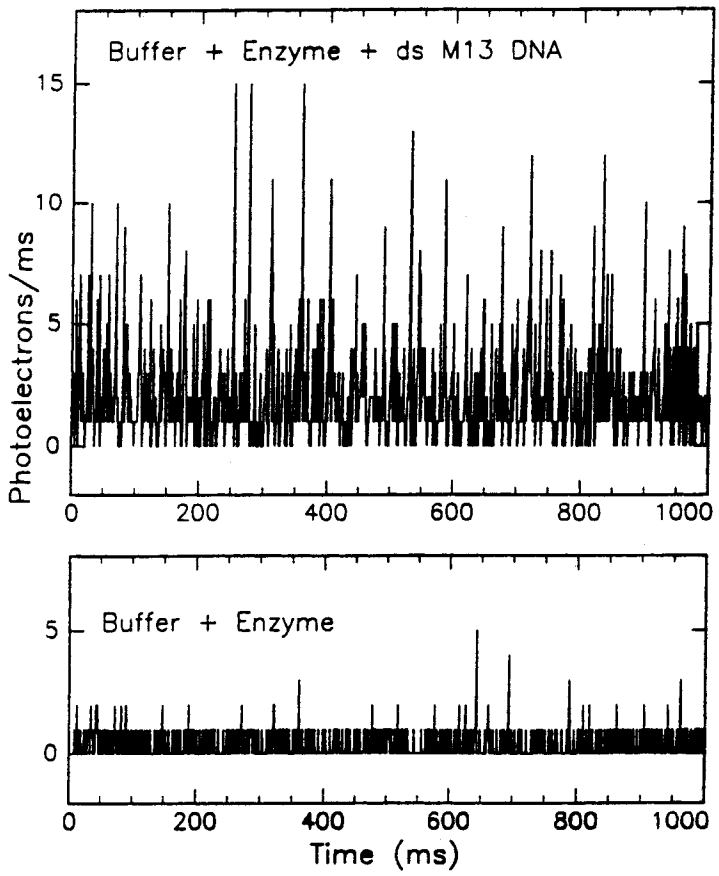

FIG.3. Detection of single TRITC-dCMP's cleaved from fragments of ds M13 DNA anchored ~ 50 μm upstream from the detection volume.

the center of the flow cell in an optical trap formed by focusing ~ 250 mW of 1064 nm light to a beam waist of < 1 μm.

Cleavage and detection of fluorescently labeled DNA in flow[5,9]

Approximately 50 fluorescently labeled (TRITC-dCTP) DNA fragments were attached to a 10 μm bead suspended in the flow cell. Exo III, a non processive exonuclease, (300 units/ml in 50 μM Tris-HCl buffer, pH8, 5 mM $MgCl_2$) was switched into the sheath stream at t = 50 sec. After ~ 300 seconds, the active buffer (containing Exo III) reached the microsphere and began to digest the DNA. In Fig. 4, the signal from B to B' results from Exo III releasing TRITC-dCTP from the DNA on the bead. The signal decays as smaller strands were digested completely and no longer contributed to the signal.

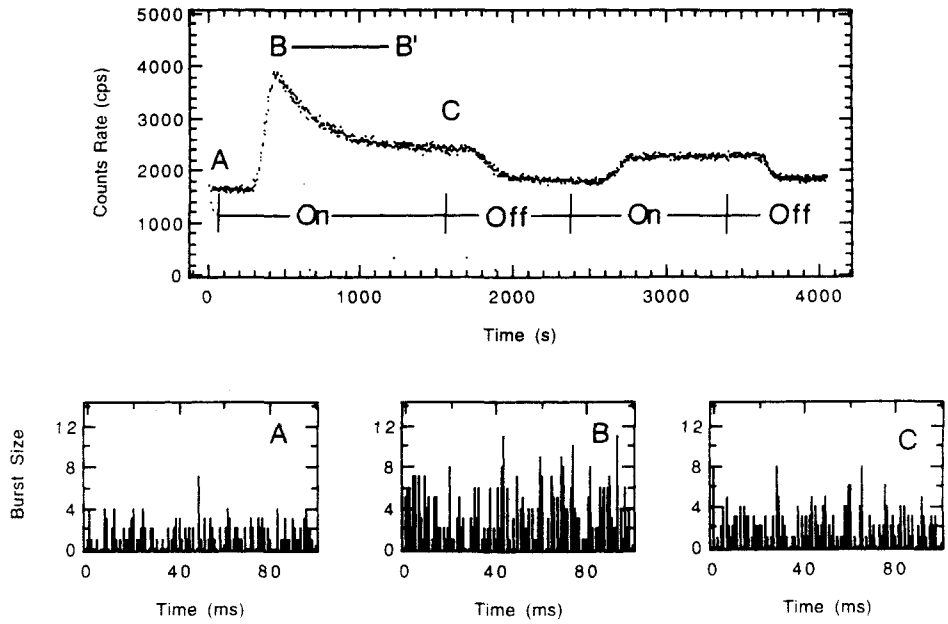

FIG.4. Detection of single, fluorescent nucleotides enzymatically cleaved from DNA fragments mounted in our apparatus. Top - gated count rate; bottom - bursts from single fluorescent molecules.

At ~ 1600 seconds, the active buffer (containing EXO III) was switched off and only pure buffer flowed through the flow cell. At ~ 2400 seconds the active buffer was again switched into the flow stream. Gated count rate (top) and photon bursts from different time points (bottom) are shown in Fig. 4. The bursts shown in Panels A and C are from fluorescent impurities present in the pure buffer solution and buffer plus enzyme. The additional bursts shown in Panel B are from single, fluorescently labeled nucleotides cleaved from the DNA by the enzyme. The enzymatic cleavage rate extracted from data was approximately 3 bp per second, in good agreement with the enzymatic digestion rate reported for Exo III on native DNA under static conditions at room temperature[13].

SUMMARY

Considerable progress has been made on our approach to DNA sequencing. We have demonstrated: labeling of long strands of DNA with two and three different nucleotides tagged with fluorescent dyes; selection and suspension of single fragments of DNA, exonuclease cleavage of labeled DNA in flow; and detection of single, fluorescently labeled nucleotides cleaved from DNA fragments anchored in a flow stream.

We are currently exploring other exonucleases to increase the cleavage rate. Future work will demonstrate the detection of single fluorescent labeled nucleotides cleaved from a single DNA fragment mounted in our apparatus. We will then move to the detection and identification of two different nucleotide types cleaved from a single DNA fragment. Note, while we intend to sequence by labeling all four nucleotide types, the complete sequence can be derived from all combinations of two color sequencing.[14]

ACKNOWLEDGMENT

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