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TITLE DNA SEQUENCING BY A SINGLE MOLECULE DETECTION OF LABELED NUCLEOTIDES SEQUENTIALLY CLEAVED FROM A SINGLE STRAND OF DNA

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DNA sequencing by single molecule detection of labeled nucleotides sequentially cleaved from a single strand of DNA

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ABSTRACT

We are developing a laser-based technique for the rapid sequencing of large DNA fragments (several kb in size) at a rate of 100 to 1000 bases per second. Our approach relies on fluorescent labeling of the bases in a single fragment of DNA, attachment of this labeled DNA fragment to a support, movement of the supported DNA into a flowing sample stream, sequential cleavage of the end nucleotide from the DNA fragment with an exonuclease, and detection of the individual fluorescently labeled bases by laser-induced fluorescence.[1] See Fig. 1 for an overview of our approach.

1. STATUS

1.1. Synthesis of fluorescent DNA[2]

The DNA to be sequenced is copied by a DNA polymerase in the presence of fluorescent analogs of the four canonical deoxynucleoside triphosphates (dNTPs). Each different dNTP contains a different fluorophore attached to the base moiety by a linker arm.

In initial studies, we have examined the activity of nine different polymerases and reverse transcriptases with several modified pyrimidine nucleotides containing either rhodamine or fluorescein and linker arms differing in length and structure. Using an M13 phage template (7.2 kb in size), modified T7 or T5 DNA polymerases will synthesize DNA greater than 7 kb in size in reactions in which either dCTP or dTTP is completely replaced by a cognate rhodamine- or fluorescein-labeled molecule. When both rhodamine-dCTP and fluorescein-dUTP are present in the reactions, DNA in the 5-7 kb size range is synthesized by modified T7 DNA polymerase.

The fidelity of the incorporation reaction is currently under study.

1.2. Exonuclease cleavage of labeled DNA strands

Preliminary studies suggest that nucleases such as E. coli exonucleases I and III, and the exonuclease activities of DNA polymerases will digest fluorescein- and rhodamine-labeled DNA. For example, the rate of digestion by the exonuclease activity of T7 DNA polymerase appears to be at least ten bases per second on fluorescent DNA in solution.

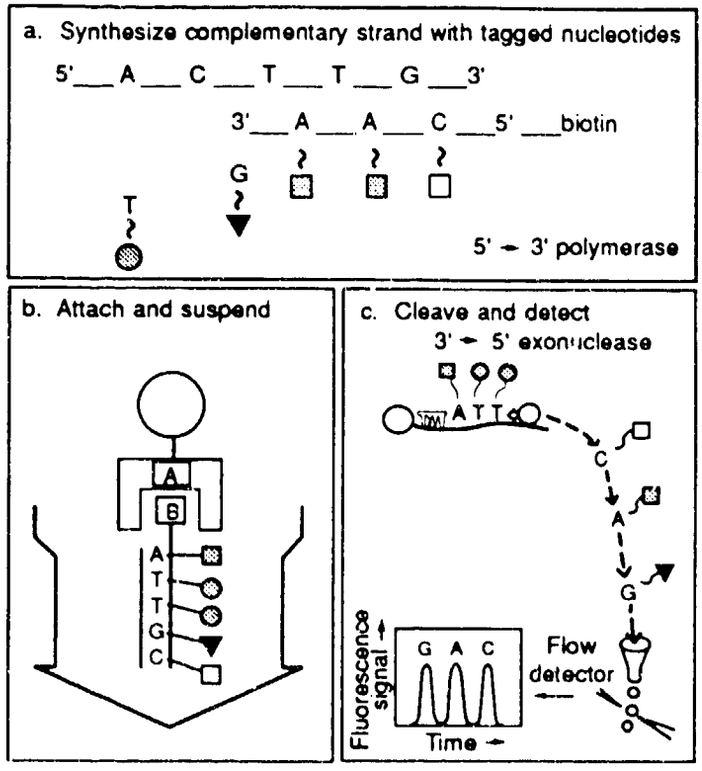

Fig. 1. Sequencing DNA by detection of fluorescently labeled nucleotides from a single DNA molecule. (a) Complementary-strand synthesis with differently tagged nucleotides. (b) Binding fluorescent biotinylated DNA molecules to avidin-coated microspheres, and immobilizing spheres in a sample chamber. (c) Release and detection of the tagged nucleotides. From Reference 1.

Our current studies are directed towards measuring rates of digestion of fluorescent DNA bound to solid supports and the analysis of the processivity of nuclease activities.

1.3. Suspension of single fragments of DNA in a flowing stream

We have attached one end of λ DNA fragments (48.5 kb in length, biotinylated at one end) to the tip of a cleaved, quartz fiber. The free end of the DNA was subsequently attached to a 3-μm diameter, streptavidin-coated, magnetic microsphere. Observation of the microsphere as it was manipulated by an external magnet signaled the presence of the DNA, and provided a measurement of the extension length, as summarized in Fig. 2.[3] Single fragments of DNA stained with an intercalating dye have been detected in a flow cytometer.[4] We are in the process of developing procedures for attaching a small number of fragments of DNA to a microsphere. The microspheres containing only a single fragment of DNA will then be selected by flow cytometry.

1.4. Single molecule detection

We have made considerable progress in single molecule detection. Picosecond excitation with time gated detection is used to discriminate fluorescence from Rayleigh and Raman background. We can

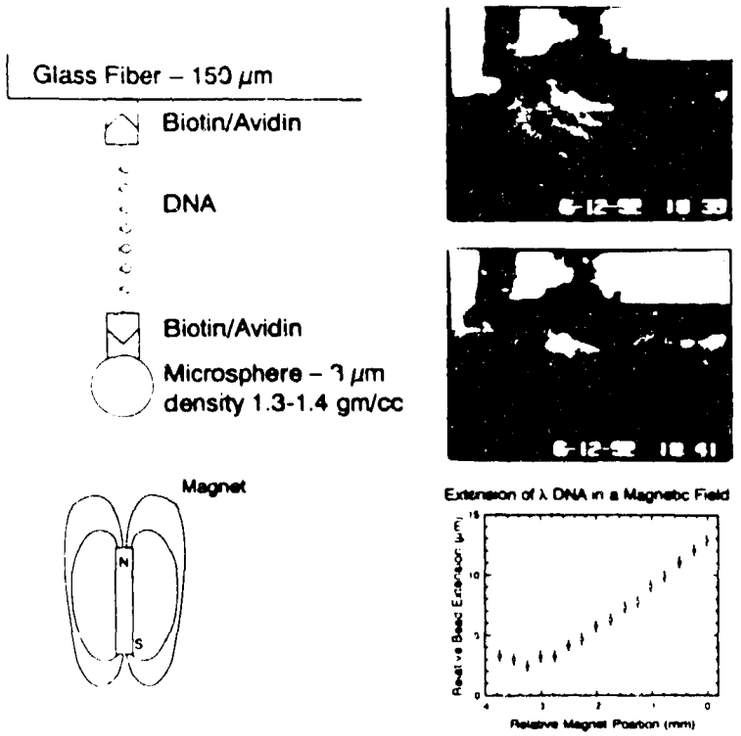

Fig. 2. Attachment and suspension of a single strand of λ DNA.

observe photon burst signals from single molecules in raw, unfiltered data in real time.[5] A short segment of raw data is shown in Fig. 3.[5] The horizontal axis denotes detected event number and the vertical axis the amount of time between successive photoelectrons. A fluorescence burst is evidenced by a series of short intervals between events. Analysis of the times between excitation and photoelectron arrival is used to measure the lifetime of single molecules.[5] Both the lifetime of a single molecule and the distribution of lifetimes from measurements on many single molecules are shown in Fig. 4. Lifetimes as well as wavelengths of fluorescence emission can be used to distinguish among different fluorescent tags.

2. SUMMARY

Considerable progress has been made towards our goal of developing a DNA sequencing technique with a projected sequencing rate of 100 to 1000 bases per second on DNA fragments greater than 10 kb in length. We have demonstrated labeling of long strands of DNA with two different colored tags, exonuclease cleavage of fluorescently labeled DNA, and single molecule detection. We are making progress in selecting and suspending single fragments of DNA and we hope to demonstrate sequencing within a year.

3. ACKNOWLEDGEMENTS

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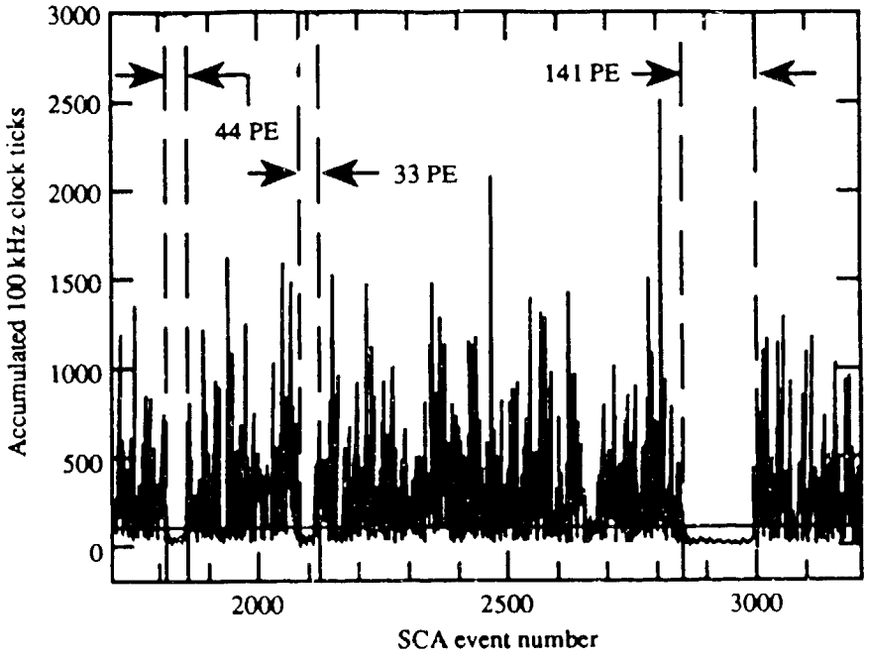

Fig. 3. Photon bursts from Rhodamine-110. From Reference 5.

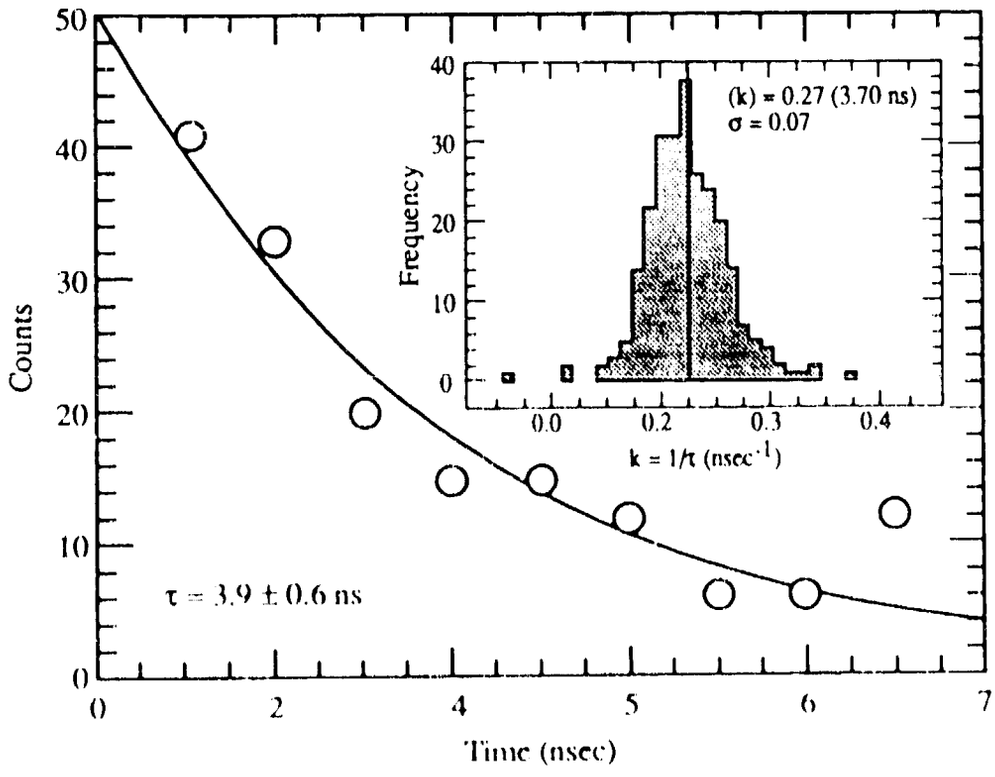

Fig. 4. Lifetime measurement on a single molecule of Rhodamine-110.

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