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Direct Imaging of DNA Motif Sequences With Encoded Nanoparticles

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Abstract— **We present a method for encoded tagging and imaging of short nucleic acid motif chains (oligomotifs) using selective hybridization of heterogeneous Au nanoparticles. The resulting encoded nanoparticle string is thus representative of the underlying motif sequence. Since the nanoparticles are much more massive than the motifs; the motif chain order can be directly observed using scanning electron microscopy. Using this technique we demonstrate direct sequencing of oligomotifs in single DNA molecules consisting of four 100-bp motif chains tagged with four different types of nanoparticles. The method outlined is a precursor for a high density direct sequencing technology.**

I. Introduction

Recent advances in high-throughput sequencing technology permit the reading of entire genomes in hours to days [1,2]. Sequencing in many of these systems is based on detection of massively parallel sequential addition reactions applied to a library of genomic oligos. The readout mechanism is often fluorescence based [3], but recently faster and less expensive electrochemical readout has been introduced [4]. In this paper we introduce a new type of physical readout based on the imaging of DNA-conjugated nanoparticles.

Nanoparticles conjugated with DNA are primarily used to form multidimensional structures [5]. Their nanometer size gives rise to high reactivity and beneficial, stable physical properties (electrical, electrochemical, optical, and magnetic) that are chemically alterable. Au and Ag nanoparticles also have very high light-scattering power. These sequence-specific nano-assemblies have various applications in nanofabrication and biomedical detection. Most detection techniques involve short tagged DNA probes selectively hybridized to longer target DNA motifs. Various tagging methods can be used for detecting the hybridization. However, the potential of nanoparticles in the field of DNA sequencing is yet to be explored. Over the past two decades the most dominant method has been optical fluorescence via molecular probes [6,7]. While the utilization of bright molecular fluorophores conjugated to DNA probes enables the detection of hybridization even in single molecules, fluorophore tags have inherently poor spatial resolution. Conventional optical microscopes cannot image the fluorophore emission with resolution better than about a quarter of the emission wavelength [6,7], an equivalent of about 500-1000 bases. In general, the utilization of molecular fluorophores for single molecule imaging presents many delicate chemical and photo-physical challenges associated with quenching, photo-bleaching, short fluorescence lifetimes, imaging optics and camera noise.

Nanoparticles can be a more robust and stable alternative to flurophores. Typically these nanometer-sized particles are conjugated with oligonucleotide detection probes and introduced into a solution of the target nucleotide. The target attachment is selective due to the property of the conjugated probe [8-10]. Nanoparticles are quench resistant and can generate very high signal intensities (a 60 nm Au particle is equivalent to 3.3 x 10^5 fluorescein molecules [5]) and the attachment of biomolecules like DNA or antibodies to these nanoparticles does not affect their physical properties. In contrast to molecular fluorophores, nanoparticle tags are easily imaged and localized by ordinary scanning electron microscopy (SEM) which has intrinsically a much higher resolution than any optical imaging technique. Therefore nanoparticles are good tag candidates for applications where the detection of the location, relative order and sequence of tags is needed [11-14]. In this paper we demonstrate the use of an encoded set of Au nanoparticle tags to directly image oligomotif sequences in single DNA molecules. Binding of multiple sequence-specific tags was realized on both surface-bound and free solution oligomotif molecules. The methodology represents a precursor of a new physical sequencing method.

II. Encoded Tagging of Oligo Motifs

In order to demonstrate encoded tagging of motif sequences, a series of linear oligomotif vectors were first constructed. A set of different-sized Au-NP probes were next prepared and hybridized to the vectors. This is followed by scanning electron microscopy imaging of the resulting NP-tagged oligomotif constructs. These experiments were carried out on both surface-bound and free solution motif vectors using the protocols discussed below.

A. Immobilization of Target Oligomotifs

Immobilization of the oligomotifs to a surface is desirable in order to produce long linear structures that are easy to read by combing techniques [14-16]. Oligonucleotide

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attachment to SiO_2 was done using Mercaptopropyltrimethoxysilane (MPTMS) [17]. The MPTMS layer with a thiol terminated surface serves as a coupling agent between the substrate and DNA. Oxidized silicon wafers treated with MPTMS have a monolayer with exposed thiol end groups (SH) which react with Acrydite™ modified oligonucleotides. The formation of a monolayer of MPTMS on the substrate surface with thiol head groups is important. An optimum concentration of MPTMS (5mM) was used as higher concentrations would lead to disordered and disoriented surface with lesser thiol headgroups on the uppermost surface [18]. The SiO_2 surface was treated with 5mM MPTMS for 48 hrs and washed with deionized water, acetone and methanol.

Successful formation of MPTMS monolayers is followed by the attachment DNA oligomotifs to the surface. The attachment of a single long ssDNA (>1000 b) is difficult. Instead, the long oligomotif is ligated to a short Acrymide (5`) modified oligonucleotide anchor which is already immobilized to the surface. The long double stranded DNA was denatured at a high temperature (95ºC) and tagged as discussed in the section below.

B. Nanoparticle Tagging

Biotinylated single strand primers complimentary to specific segments in the long target oligomotif were selected. The primers were designed to counter self looping and non specific binding. The nanoparticles are attached either before or after the c-DNA hybridization depending on the application as discussed in sections 3.1 and 3.2. As per scheme 1, after the probe hybridization reaction, Streptavidin conjugated nanoparticles are allowed to attach to the biotinylated c-DNA. Nanoparticles of different sizes are hence attached to a single long target oligomotif. The nanoparticles selected are large enough for SEM imaging but small enough to avoid overcrowding and coagulation. In this study we used Au nanoparticles 30, 20, 10 and 5 nm in diameter, designated as P1, P2, P3 and P4 correspondingly.

III. EXPERIMENTS AND RESULTS

A. Homogeneous-NP-tagged Oligomotif : Scheme I

In order to determine the feasibility of the nanoparticle tagging scheme, we first attempted tagging the oligomotif with nanoparticles of uniform size. First we tagged a single motif with a single nanoparticle as follows. Acrydite modified dsDNA consisting of 78 bp motifs was first ligated with 200bp dsDNA. The ligated DNA (278bp) was then immobilized on MPTMS treated SiO_2. The immobilized DNA was next heated at 95˚C to denature. A biotinylated ssDNA probe (112bp) was next hybridized to a single motif in the immobilized DNA. Subsequently, the samples were immersed in a solution of Streptavidin conjugated P1 Au nanoparticles (30 nm) overnight. The samples were next washed three times in TE buffer (pH 7.6) to flush out non-specific bound nanoparticles. Figure 2 shows SEM image of a MPTMS treated SiO_2 surface after DNA immobilization and subsequent P1 nanoparticle attachment. As expected a single nanoparticle binds to each immobilized DNA molecule as shown in Figure 1

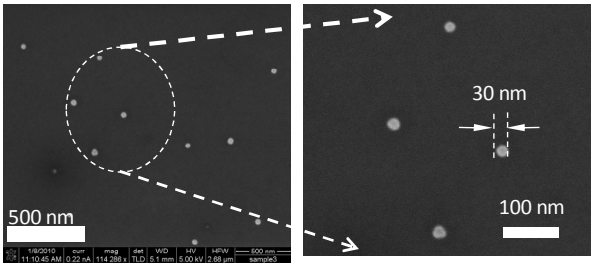

Figure 1: SEM images of MPTMS-immobilized oligos tagged with single P1 (30 nm) nanoparticles.

Next we tagged to multiple motifs with homogeneous nanoparticles as shown in Figure 2. Linearized pET-3a DNA was first ligated to a short ds-oligo immobilized on SiO_2 surface.

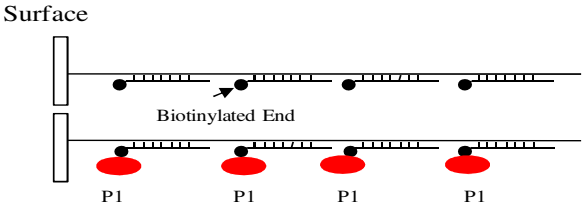

Figure 2: Homogeneous NP-tagging of DNA motifs

The DNA was next denatured and a set of short biotinylated single-stranded probes, complimentary to a few pET-3a motifs, was hybridized to the longer strand on the surface. The samples were next dried for SEM imaging. Figures 3a-b show the simultaneous tagging of three separate 100bp motifs of pET-3a, separated by 100 bp using P1 nanoparticles. Figure 3c shows the linkage of seven P1 nanoparticles to seven sequential 100bp motifs of pET-3a. These preliminary experiments indeed demonstrate the correct imaging of probe hybridization to a known number of motifs using nanoparticles.

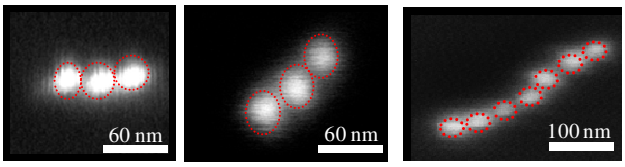

Figure 3: SEM images of P1 Au nanoparticle tags linked to specific pET-3a motifs.

B. Encoded NP-tagged Oligomotifs: Scheme II

In order to link nanoparticle tags of different sizes to linearized pET-3a motifs a different scheme was adopted. The different nanoparticles are first conjugated with motif-

specific ss-DNA probes and hybridized later. This is the only strategy that produces encoded chains of nanoparticle tags to oligomotifs. Encoded tagging is important for identification of motif order and motif sequencing, and this procedure can be utilized as a precursor in DNA sequencing applications. Several combinations of nanoparticles and motif probes that were used are shown in Figure 4. If the encoded tagging is successful, the resulting nanoparticle chain pattern specified by its particle index vector $\vec{p} = (i, j, \cdots, n)$ corresponds exactly to the oligomotif sequence. The ss-DNA probes complimentary to one of the several target-motifs were first linked with nanoparticles of the specific size required for each pattern. The sequences of the Motifs used are given in Table 1 below.

Motif 1	CCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGA CGATGAGCGCATTGTTAGATTTCATACACGGTGCCTGACTG CGTTAGCAATTTAACTGTG
Motif 2	CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCT GAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGG TCTTGAGGGGTTTTTTGCTG
Motif 3	AATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGG CCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCT CATGAGCCCGAAGTGGCGAG
Motif 4	TGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTG ACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCG CAAGCGACAGGCCGATCAT

Table 1: Sequence of Motifs used in the experiment

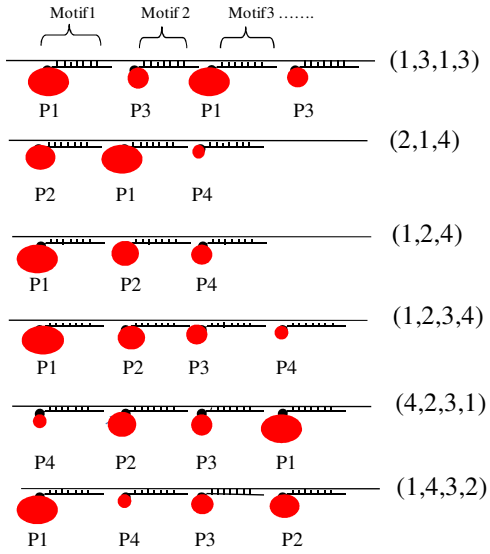

Figure 4: Some of the patterns with which biotinylated ss-DNA probes are coupled to different Au nanoparticles were hybridized to linearized pET-3a motifs. (Particles P1, P2, P3 and P4 are 30, 20, 10 and 5 nm in diameter respectively)

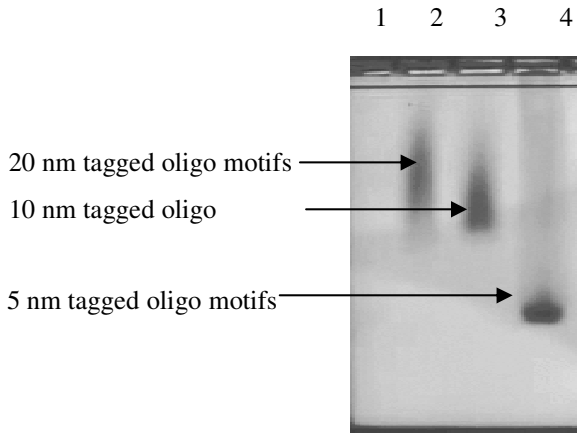

Figure 5: Agarose gel (2%) electrophoresis of nanoparticle tagged oligo motifs

For instance, to generate the particle index vector (2, 1, 4), the ss-DNA probes for motifs 1, 2 and 3 were linked to nanoparticles-P2, P1 and P4 respectively. However, to generate vector (1, 2, 4), probes for motifs 1, 2 and 3 were linked with nanoparticles-P1, P2 and P3 respectively.

The corresponding nanoparticles tagged probes were formed as follows. The relevant probes were added into aqueous dispersions of the corresponding Streptavidin conjugated Au nanoparticle. The mixture was incubated at room temperature for 24 hrs. Subsequently, the aqueous solution of NaCl (5 mol/L, 50 µL) was added into the mixture solution. After 24 h, an additional 50 µL NaCl (5 mol/L) was added. After further incubation for 24 h the nanoparticles were centrifuged for 10 min at 10,000 rpm. The precipitate was washed three times with 0.3 mol/L NaCl, 10 mmol/L phosphate buffer (pH 7.0, referred as 0.3 mol/L PBS) to remove the excess non-conjugated probes [19]. The encoded nanoparticle-tagged probes were resolvated with nuclease-free water and added to a solution containing linearized pET-3a for hybridization. Samples were next dried and imaged using a scanning electron microscope.

Figure 5 shows the agarose gel electrophoresis of nanoparticle-ssDNA conjugates. The electrophoresis was performed for 25 minutes to separate the oligomotifs conjugated with nanoparticles. The distinct bands can be identified from the figure. Lane 2, 3, 4 are 20, 10, 5 nm tagged oligomotifs respectively. As expected, the motifs conjugated with smaller nanoparticles were mobilized faster than the bigger ones (5 >10 >20 nm). Lane 1 was loaded with motifs conjugated with 30nm nanoparticles; however it did not mobilize in 2% gel.

The vectors realized are shown in Figures 6, 7 and 8 below. Figure 6 shows SEM images of a few resulting encoded particle vectors formed using nanoparticles of *two* different sizes. Figure 7 shows SEM images of realized encoded particle vectors formed using *three* different nanoparticles. Figure 8 shows SEM images of realized encoded particle vectors formed using *four* different

nanoparticles. All the vectors shown in Figure 4 were realized. In particular, the realization of four-particle encoded vectors which are clearly imaged by conventional SEM techniques is of high technological relevance. Such tagging techniques may potentially lead to new nanoparticle-based single-molecule sequencing methodologies.

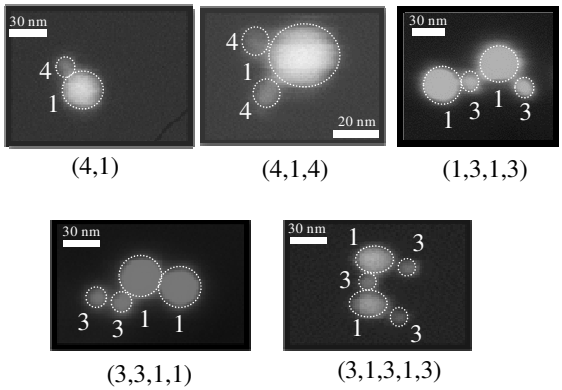

Figure 6: SEM images of some of the encoded particle vectors generated using *two* different nanoparticles.

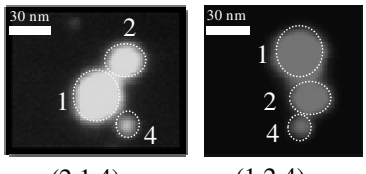

Figure 7: SEM images of encoded particle vectors generated using *three* different nanoparticles.

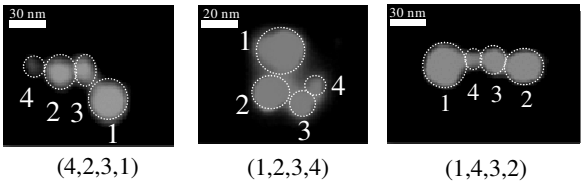

Figure 8: SEM images of encoded particle vectors generated using *four* different nanoparticles.

IV. CONCLUSION

Nanoparticles of various sizes (30, 20, 10 and 5nm) were simultaneously linked to linearized pET-3a oligomotifs to produce encoded tag vectors representative of the motif sequences. The resulting nanoparticle vectors and the underlying oligomotif sequences are directly observable using scaning electron microscopy. The experimental SEM images clearly confirm that expected nanoparticle vectors can be materialized by interchanging conjugated nanoparticle and motif probe combinations. The applicability of the encoded tagging technique to new nanoparticle-based DNA sequencing methods is currently under study.

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