Direct imaging of DNA motif sequences with encoded nanoparticles

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We present a method for encoded tagging and imaging of short nucleic acid motif chains (oligomotifs) using selective hybridization of heterogeneous Au nanoparticles (Au-NP). The resulting encoded NP string is thus representative of the underlying motif sequence. As the NPs 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-nt motif chains tagged with four different types of NPs. The method outlined is a precursor for a high density direct sequencing technology.

Introduction

The development of techniques for sequence-specific detection of DNA has progressed greatly in recent years. 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. Over the past two decades the most dominant and most sensitive method has been optical fluorescence via molecular probes [1,2]. 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 [1,2], an equivalent of about 500–1000 bases. Imaging below this limit in the conventional far field (Abbe’s limit) sense is not possible, but high-resolution localization of the emission peaks within 50–100 bases [2–6] has been reported using near field microscopy or very low-noise cameras with long integration times. 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.

As alternatives to molecular fluorophores, more robust and stable physical tags such as nanoparticles (NPs) have been recently developed. 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 because of the property of the conjugated probe [7–10]. NP tags have unique properties. 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 NPs also have very high light-scattering power [11]. NPs are quench-resistant and can generate very high signal intensities (a 60 nm Au particle is equivalent to 3.3 × 10^5 fluor-fluorescin molecules [11]) and the attachment of biomolecules such as DNA or antibodies to these NPs does not affect their physical properties. In contrast to molecular fluorophores, NP 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, NPs are good tag candidates for applications where the detection of the location, relative order and sequence of tags is needed [12–14]. In this paper we demonstrate the use of an encoded set of Au-NP 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. While the emphasis of the paper is on sequencing of motifs by physical means, this process, however, can be used as a precursor method for de novo sequencing as follows. First a library of short genomic oligos is expanded so that each base is converted to a distinct motif. This
can be done using conventional methods described by both Lexow [16] and McNally [17]. The NP-tagging process can then be used to read out the converted oligo directly without further use of fluorescence markers or long sequential base ligation sequencing methods [15].

Materials and methods
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 SEM imaging of the resulting NP-tagged oligomotif constructs. The vector pET-3a was purchased from New England Biolabs, custom-modified ss-DNA samples were ordered from IDT DNA (http://www.idtdna.com) and NPs were purchased from Nanopartz (http://www.nanopartz.com). All the chemicals used were of molecular biology grade and were purchased from Sigma–Aldrich, USA. These experiments were carried out on both surface-bound and free solution motif vectors using the protocols discussed below. Figure 1 shows a schematic representation of the type of NP-tagged motif vectors immobilized to a surface used in this study. FEI NanoNova SEM, having a resolution of 2 nm, was used to image the samples. The SEM was operated in vacuum mode and a low-voltage high-contrast detector was used.

**Immobilization of target oligomotifs**
Immobilization of the oligomotifs to a surface is desirable to produce long linear structures that are easy to read by combing techniques [18–20]. Oligonucleotide attachment to SiO$_2$ was done using mercaptopropyltrimethoxysilane (MPTMS) [21]. 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 (5 mM) was used, as higher concentrations would lead to disordered and disoriented surface with lesser thiol headgroups on the uppermost surface [22]. The SiO$_2$ surface was treated with 5 mM MPTMS for 48 h 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 ss-DNA (>1000 nt) was difficult. Instead, the long oligomotif is ligated to a short acrylamide (5') modified oligonucleotide anchor, which is already immobilized to the surface. The long double stranded DNA was denatured at a high temperature (95°C) and tagged as discussed in the section below.

**Nanoparticle tagging**
Biotinylated single strand primers complementary to specific segments in the long target oligomotif were selected. The primers were designed to counter self-looping and nonspecific binding. The NPs are attached either before or after the c-DNA hybridization depending on the application as discussed in Sections ‘Homogeneous-NP-tagged oligomotif’ and ‘Dissimilar NP-tagged oligomotifs’. As per

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**FIGURE 1**
Scheme used for immobilization and NP-tagged hybridization of oligomotifs.

**FIGURE 2**
SEM images of MPTMS-immobilized oligos tagged with single P1 (30 nm) nanoparticles.
scheme I, after the probe hybridization reaction, streptavidin-conjugated NPs are allowed to attach to the biotinylated c-DNA. NPs of different sizes are hence attached to a single long target oligomotif. The NPs selected are large enough for SEM imaging but small enough to avoid overcrowding and coagulation. In this study, we used Au-NPs 30, 20, 10 and 5 nm in diameter, designated as P1, P2, P3 and P4 correspondingly; 100 nt DNA (0.35 nm x 100 = 35 nm) was chosen so that the length of the DNA strand is longer than the diameter of the largest NP (30 nm). Shorter oligomers could be used but at the expense of smaller NPs and higher SEM resolution. There is a tradeoff between the size of the motif oligomer length and the maximum size of NP that can be used. Naturally smaller motifs can only accommodate smaller NPs which could be difficult to image.

**Homogeneous-NP-tagged oligomotif**

Acrydite-modified dsDNA consisting of 78 bp motifs was first ligated with 200 bp dsDNA. The ligated DNA (278 bp) was then immobilized on MPTMS-treated SiO$_2$. The immobilized DNA was next heated at 95°C for denaturation. A biotinylated ss-DNA probe (112 nt) was next hybridized to a single motif in the immobilized DNA. Subsequently, the samples were immersed in a solution of streptavidin-conjugated P1 Au-NPs (30 nm) overnight. The samples were next washed three times in Tris-EDTA (TE) buffer (pH 7.6) to flush out nonspecific bound NPs.

**Dissimilar NP-tagged oligomotifs**

The relevant probes were added into aqueous dispersions of the corresponding streptavidin-conjugated Au-NP. The mixture was incubated at room temperature for 24 h. 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 NPs 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 phosphate buffer saline (PBS)) to remove the excess nonconjugated probes [23]. The encoded NP-tagged probes were resolivated with nuclease-free water and added to a solution containing linearized pET-3a for hybridization. Samples were next dried and imaged using a SEM.

**Experiments and results**

**Homogeneous-NP-tagged oligomotif: scheme I**

To determine the feasibility of the NP-tagging scheme, we first attempted tagging the oligomotif with NPs of uniform size. We
tagged a single motif with a single NP as described in Section ‘Materials and methods’, scheme I. Figure 2 shows SEM image of a MPTMS-treated SiO₂ surface after DNA immobilization and subsequent P1 NP attachment. As expected a single NP binds to each immobilized DNA molecule as shown in Fig. 2. The blank samples (with no DNA attached) did not retain NPs after it were washed three times in TE buffer (pH 7.6).

Next we tagged to multiple motifs with homogeneous NPs as shown in Fig. 3. Linearized pET-3a DNA was first ligated to a short ds-oligo immobilized on SiO₂ surface. The DNA was next denatured and a set of short biotinylated single-stranded probes, complementary to a few pET-3a motifs, was hybridized to the longer strand on the surface. The samples were next dried for SEM imaging. Figure 4a and b shows the simultaneous tagging of three separate 100 nt motifs of pET-3a, separated by 100 nt using P1 NPs. Figure 4c shows the linkage of seven P1 NPs to seven sequential 100 nt motifs of pET-3a. These preliminary experiments indeed demonstrate the correct imaging of probe hybridization to a known number of motifs using NPs.

**Encoded NP-tagged oligomotifs: scheme II**

To link NP tags of different sizes to linearized pET-3a motifs a different scheme was adopted. The different NPs are first conjugated with motif-specific ss-DNA probes and hybridized later. This is the only strategy that produces encoded chains of NP tags to oligomotifs. Encoded tagging is important for identification of

![Image](https://via.placeholder.com/150)

**FIGURE 6**

(a) SEM images of some of the encoded particle vectors generated using two different nanoparticles.

(b) SEM images of encoded particle vectors generated using three different nanoparticles.

(c) SEM images of encoded particle vectors generated using four different nanoparticles.
motif order and motif sequencing, and this procedure can be utilized as a precursor in DNA sequencing applications. Several combinations of NPs and motif probes that were used are shown in Fig. 5. If the encoded tagging is successful, the resulting NP chain pattern specified by its particle index vector $\bar{\beta} = (i, j, \ldots, n)$ corresponds exactly to the oligomotif sequence.

The ss-DNA probes complementary to one of the several target-motifs were first linked with NPs of the specific size required for each pattern as described in Section ‘Homogeneous-NP-tagged oligomotif’. For instance, to generate the particle index vector (2, 1, 4), the ss-DNA probes for motifs 1, 2 and 3 were linked to NPs P2, P1 and P4, respectively. However, to generate vector (1, 2, 4), probes for motifs 1, 2 and 3 were linked with NPs-P1, P2 and P3, respectively. The sequences of the Motifs used are given in Table 1. The vectors realized are shown in Fig. 6a–c.

Figure 6a shows SEM images of a few resulting encoded particle vectors formed using NPs of two different sizes. Figure 6b shows SEM images of realized encoded particle vectors formed using different NPs. Figure 6c shows SEM images of realized encoded particle vectors formed using four different NPs. All the vectors shown in Fig. 5 were realized.

In particular, the realization of four-particle encoded vectors, which are clearly imaged by conventional SEM techniques, is of high technological relevance because of its direct applicability to sequencing. For example, if the NP-tagged fragments are ordered on the substrate, an information packing density of 1 kb/µm² could be reached, with a capacity of 100 Gb/cm². With SEM scanning rates of 2 µm × 2 µm in 20 ns, the substrate genomic information could be read out at a rate of 720 Mb/s, about two times faster than Life Technologies state-of-the-art SOLID™ system (~300 Mb/h). Because of the information transfer from an organic molecule to essentially a NP chain, our technique could produce a solid-state memory imprint of a genome. Work is in progress toward transferring the NP patterns to a silicon substrate for atomic force microscopy (AFM) or electronic readout.

The technology offers a robust alternative to the fluorophore-based approach and, to a large extent, circumvents the problems associated with fluorescence (quenching, low resolution, among others) Right now the limitation of this method is the time consumed in sample preparation and scanning. Our throughput right now is limited by two things: (1) sample density and (2) SEM scan speed. Sample density can be increased considerably by increasing the sample concentration, combing and surface immobilization of one end of the oligomotif chain. The SEM speed can be significantly increased if we use a different type of inline inspection SEM that is commonly used for automated SEM inspection of semiconductor chips.

The main objective of this study was the demonstration of direct readout of NP-tagged DNA as design polymers as indicated by our results. However, incomplete vectors were also generated. Currently, the incomplete vector generation is between 30 and 60%. Work is in progress to determine the exact repeatability and reproducibility of the experiment.

**Conclusion**

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

**References**