DNA stretching on functionalized gold surfaces

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ABSTRACT

We describe a method for anchoring bacteriophage λ DNA by one end to gold by Au – biotin – streptavidin – biotin – DNA bonds. DNA anchored to a microfabricated Au line could be aligned and stretched in flow and electric fields. The anchor was shown to resist a force of at least 11 pN, a linkage strong enough to allow DNA molecules of chromosome size to be stretched and aligned.

INTRODUCTION

The microscopy of DNA is an important tool for analyzing structure. Highly resolved images have been obtained by electron microscopy (1–4), scanning probe microscopy (5–8) and fluorescence microscopy (9–14). In the first two techniques, the DNA must be fixed to a surface in order to achieve high resolution. In fluorescence microscopy, the molecule must be confined within the focal plane in order to image the entire molecule. However, in all of these techniques, the image of very large DNA molecules is, more often than not, a projection of a random coil onto a surface, which makes the topology of the molecule difficult to interpret.

This problem has been partially solved by taking advantage of induced shear forces during preparation of DNA for electron microscopy (1) and ^3H radioautography (15). More recently, the ends of individual λ DNA molecules have been selectively derivatized so that individual molecules can be manipulated and studied by fluorescence microscopy (10, 16). Individual chromosome-sized yeast DNA molecules have also been imaged in agarose and followed during restriction digestion (17). These methods all rely on DNA bound or hooked at randomly distributed sites. For many analyses, however, it is important to anchor, align, and stretch many DNA molecules specifically and at high concentration to well defined microstructures.

Here we show how λ DNA monomers and concatemers, tethered to Au microstructures, can be stretched in a flow or electric field. DNA was tethered to a Au substrate by a biotin–streptavidin–biotin–Au linkage. The biotin–Au complex was constructed by exploiting the observation that alkane thiols (18–20), and also thiol derivatives of biotin (21), can self-assemble on Au surfaces. Streptavidin was then bound to the biotinylated Au surface, and reacted with λ DNA biotinylated at the left end (Fig 1). Because of the high biotin–streptavidin binding constant ($K_d \sim 10^{-15}$ M) (22–25), this bond resists the stretching force necessary to straighten DNA molecules longer than 250 000 base pairs (250 kbp). The stability of the binding,

the stretching of the DNA in a fluid flow, and its alignment in an electric field was then studied by confocal microscopy.

MATERIALS AND METHODS

Surface preparation

In the procedures described below the reagents are specified by manufacturer, or were 'Reagent' or 'HPLC' grade (most solvents). The water was distilled and deionized (resistance > 16 M Ω), and filtered through a 0.45 μm filter. Glass surfaces and silicon wafers with a thermally grown 200 nm oxide layer were cleaned for 1 h in a mixture of water, hydrogen peroxide, and ammonium hydroxide (20:4:4 by volume, heated to about 70°C). After the cleaning step, these surfaces were extensively rinsed with water and dried in a heated vacuum chamber. A 5 nm thick Cr layer was vapor deposited on the cleaned surface and coated with a 150 nm thick layer of Au without breaking the vacuum. The vapor deposition of both Au and Cr was performed under standard high vacuum conditions (10^{-6} Torr) at room temperature (26). After the deposition process, all samples were immediately stored in a dry and filtered argon atmosphere. For radioactive assays, pre-cleaned silicon wafers were thoroughly covered with the Cr/Au layer, and then cut into pieces of varying size. For DNA imaging, Au lines were evaporated onto pre-cleaned coverslips. Au lines wider than 1 mm were fabricated with a thin aluminum mask. Lines 5 μm wide were constructed using standard photolithographic masking techniques in combination with vapor deposition (26).

Biotinylation of Au surfaces

The Au surfaces were first cleaned using the above procedure and stored in filtered distilled water for no more than 30 min. They were never allowed to dry between treatments. The reaction solution for surface modification was freshly prepared in two steps. First, 1 mg of biotin–HPDP (Pierce) was dissolved in 100 μl N,N -dimethylformamide. Five microliters of tri- n -butylphosphine (Sigma) was then added and reacted for 5 min. The reduced solution is yellow. This mixture was next added to 4 ml of a water/methanol (1:1) solution containing the samples. A 10 min reaction time proved sufficient for this step (see below). The sample was then washed in water/methanol (1:1) and stored in PB buffer (50 mM Na_2HPO_4 , pH 7). The reduction of biotin–HPDP to biotin–HP thiol and pyridine thiol was also followed by thin layer chromatography on silica plates (Merck) in a mobile phase consisting of n -butanol/acetic acid/ H_2O (5/1/1). The two thiol products were visualized with Ellman reagent (Sigma).

To test the binding capacity of the derivatized and functionalized Au surfaces, L-[^{35}S]-cysteine (1300 Ci/mmol, New England Nuclear) and *d*-[8,9- ^3H]-biotin (50 Ci/mmol, New England Nuclear) were used. For determining the coverage of the Au surface, 0.25 cm² Au-covered silicon oxide pieces were sampled at various times during the biotinylation reaction by immersing them into a solution of 0.1 μM L-[^{35}S]-cysteine in PB buffer. The reaction was allowed to proceed for 1 h at room temperature. To determine the binding capacity of the biotinylated surface, the sample was first incubated in 0.35 μM streptavidin dissolved in PB buffer for 30 min at room temperature. After washing in PB, the sample was then immersed in 2 μM ^3H -biotin in PB buffer for 30 min at room temperature and washed again. The radioactivity of the preparation was measured by liquid scintillation spectroscopy. The radioactive surface was oriented Au side up. The counting rate was later normalized to a surface area of 1 cm², and converted to a surface concentration of bound molecules, taking into account the specific activity, counting efficiency and sample geometry. The coverage of the biotin layer was derived from the decrease in bound ^{35}S -cysteine. Assuming that the formation of the biotin layer leads in equal proportion to a decrease in the reactive surface area accessible to ^{35}S -cysteine, an $x\%$ biotin coverage can be calculated from a $(100-x)\%$ decrease of bound cysteine.

λ DNA modification

The recessed ends of λ DNA (New England Biolabs, 20 $\mu\text{g}/\text{ml}$ in polymerase buffer) were filled with the 4 deoxyribonucleoside triphosphates, biotin-16-dUTP, and digoxigenin-11-dUTP (both from Boehringer) by using T4 polymerase fragment 1 (New England Biolabs). The incubation time for each filling step was 1 h at room temperature. The DNA was then freed of unincorporated deoxynucleotides and salts with a spin filter (Centricon 30, Amicon) by replacing the solvent with TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) at least four times.

DNA concatemers were constructed by adding 400 units of T4 DNA ligase (New England Biolabs) to a 200 μl solution of 40 $\mu\text{g}/\text{ml}$ λ DNA in ligating buffer. The sample was incubated for 2 h at 37°C and then heated to 65°C for 15 min. For further end labeling, the multimers were diluted in polymerase buffer to a final concentration of 10 μg DNA/ml. The multimers were modified and purified as mentioned above, and characterized by pulsed field gel electrophoresis (27).

Binding λ DNA to surfaces

Streptavidin-coated magnetic beads (Dynal Inc.) were used for studying the extent of DNA biotinylation. Beads (0.2 mg) and 0.6 μg biotin-modified λ DNA were incubated for 30 min in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl at room temperature. Unbound DNA was washed away and the buffer replaced with 60 μl of the appropriate restriction enzyme buffer. Ten units of the restriction enzyme (New England Biolabs) were added and the sample incubated for 1 h at 37°C. Finally, the beads were separated from the supernatant in a magnetic field and the supernatant was used for gel electrophoresis (TAE buffer, 0.8% agarose, 2.5 V/cm).

λ multimers were bound to Au as follows. Streptavidin was first reacted with biotinylated Au for 30 min in a solution of 0.35 μM streptavidin in PB buffer. Following a washing step in TBE buffer (45 mM Tris, 45 mM H_3BO_3 , 2.5 mM EDTA, pH 8) the surface was reacted with 1 $\mu\text{g}/\text{ml}$ biotinylated λ DNA in TE buffer. Afterwards, the treated surface was washed carefully in

TBE for 15 min. The sample was finally mounted on a cleaned microscope slide carrying either a drop of TBE buffer or TBE buffer/0.8% low melting temperature agarose (NuSieve GTG) or TBE buffer/sucrose.

DNA imaging

Appropriately labeled λ DNA multimers (20 $\mu\text{g}/\text{ml}$) in TE were stained for 15 min in 2 μM YOPRO1 (Molecular Probes), and then diluted 20-fold into an anti-fade solution of TBE containing 5.0 μM glucose, 2 $\mu\text{g}/\text{ml}$ glucose oxidase and 15 $\mu\text{g}/\text{ml}$ catalase (all Sigma). In several experiments this solution also contained 0.8% agarose kept at 55°C. The functionalized cover slip was mounted Au side up on a pre-cleaned glass slide without being allowed to dry, and a 15 μl drop of the DNA solution was pipetted onto the surface and quickly covered with a second pre-cleaned cover slip of the same size. Two sides of the chamber were sealed with nail polish, which also fixed both cover slips to the slide. On the open ends platinum electrodes were added to apply the electric field [see for example (11)]. During the experiment the voltage was kept constant and the current was monitored. The stained DNA was imaged with a confocal microscope using the 499 nm line of an Argon/Krypton laser for excitation (Biorad MRC600).

RESULTS

Functionalizing the Au surface

We reduced the disulfide group of HPDP-biotin with tri-*n*-butylphosphine to yield a thiolated biotin derivative which could react with Au (Fig. 2). This approach is similar in principle to earlier work with alkyl thiols (19–21). The reaction was followed by thin layer chromatography, where all of the reaction products could be resolved (data not shown). The reaction was essentially complete in 5 min.

Coverage was studied indirectly by measuring ^{35}S -cysteine binding as described in Materials and Methods. Under our reaction conditions, ^{35}S -cysteine bound to a plain Au surface at a concentration of 0.4 $\mu\text{mol}/\text{cm}^2$, and this value was used to calculate the data shown in Fig. 3. Coverage increased to about 95% within minutes, reaching 98.3% coverage at 2928 min (49 h). The layer produced is probably mixed, since one of the products of biotin-HPDP reduction is pyridine thiol, which was not removed from the reduced solution, and it too should react with Au.

Figure 1. Anchoring DNA to a Au substrate: streptavidin can be bound to a self assembled layer of biotin thiol on Au. After this activation, DNA biotinylated at one end binds to the still accessible binding sites of the streptavidin layer.

To measure the binding capacity of the modified Au surface, we first bound streptavidin and then ^3H -biotin to the streptavidin. Because streptavidin is a tetramer, it can bind to a biotinylated surface and retain the ability to bind added biotin molecules. Thus bound ^3H -biotin is a direct measure of the binding capacity of the biotin-HP thiol layer. These results are also summarized in Fig. 3 as a function of incubation time. After 2 min, 2.3 pmole/cm^2 were bound in this experiment. Assuming that an average of 2 biotin molecules can bind to the bound streptavidin, the concentration of the streptavidin layer is approximately $1.2 \pm 1 \text{ pmole/cm}^2$. This represents 24% coverage, calculated from the known size of streptavidin, $5.4 \times 5.8 \times 48 \text{ nm}$ (28). Binding capacity decreases slowly over 49 h in the reaction solution, possibly because excess tributylphosphine reacts with biotin. Alternatively, pyridine thiol may displace biotin-HP thiol with time. Because of the fast initial coverage of the surface and the slow decay later in binding capacity, a 10 min incubation step is sufficient.

We next showed by competition that the binding of ^3H -biotin was specific (Table 1). Here it is evident that ^3H -biotin fails to

Figure 2. Reduction of the disulfide bond of biotin-HPDP provides the thiols used in this study.

Figure 3. Formation of the biotin thiol layer. The coverage of the surface and the binding capacity of the biotin thiol layer were studied with radioactive probes: The coverage (graph □, left axis) was calculated from the concentration of ^{35}S -cysteine bound to the partly covered surface. The binding capacity was determined by reacting streptavidin and ^3H -biotin with the layer (graph ◇, right axis).

bind if streptavidin has not first been bound to the thiol monolayer (row 2), or if the binding sites of the streptavidin are first blocked with non-radioactive biotin (row 3). This experiment also demonstrates 80% reduced binding of ^3H biotin to nonspecifically adsorbed streptavidin (row 4). Finally, ^3H biotin by itself does not bind to cleaned Au (row 5) or to SiO_2 surfaces treated with reduced HPDP.

End labeling of λ DNA

The left and right ends of λ DNA can be filled by using the Klenow fragment of T4 DNA polymerase (Fig. 4). Both ends have a single A in the overhang, the complementary base for modified dUTP. The location of A allowed us to tag the left end with biotin and the right end with digoxigenin (Fig. 4b,c). In the absence of dCTP, incorporation stops after the incorporation of biotin-dUTP (Fig. 4b). Replacing biotin-dUTP by digoxigenin-dUTP and adding dCTP labels the right end with digoxigenin (Fig. 4c). DNA labeled with biotin at the left end

Table 1. Competition experiments on gold

Biotin thiol	Streptavidin	Biotin block	^3H -Biotin	Counting rate (cpm)	%
+	+	-	+	14 700	100
+	-	-	+	100	<1
+	+	+	+	580	4
-	+	-	+	2940	20
-	-	-	+	<100	<1

Competitive binding of radiolabeled ^3H -Biotin to the streptavidin-activated biotin/Au surface confirms specific binding to the functionalized surface (no adsorption of biotin to similarly treated glass or SiO_2 was detected)

Figure 4. The complementary ends of λ DNA (a) and the directed labeling of first the left end (b) and later the right end (c). The absence of dCTP in the polymerase reaction terminates filling after incorporation of biotin-dUTP at the left end. Replacing biotin-dUTP with digoxigenin-dUTP and adding dCTP terminates left end addition, and promotes right end labeling with digoxigenin-dUTP.

Figure 5. Agarose gel electrophoresis of *StuI* digested bio- λ (a) and bio- λ -dig (b) DNA confirms the orientation of the labeling. For these experiments, the DNA was bound by the left end to streptavidin–Dynal beads, which were then washed and incubated with *StuI*. The released DNA was run on an agarose gel (0.8%, TAE). Gel (a) shows the fragments of biotin- λ DNA before (lane 1) and after (lane 2) binding DNA to the beads. The experiment was repeated with unlabeled λ DNA as a control for unspecific binding to beads (lane 3 and 4). Gel (b) shows the same experiment for bio- λ -dig DNA, (1) and (2), but in addition antidigoxigenin was added (4) which prevents the 7888 bp band from running normally in the gel. The high molecular weight faint band in lane 1, gel (b), is caused by concatemers and can be ignored. The exact sizes of the *StuI* fragments are: 19 044 bp; 12 434 bp; 7888 bp, 6995 bp.

(bio- λ) was prepared for microscopic studies; ‘bio- λ -dig’ DNA carries digoxigenin at the right end.

The specificity of the reaction was demonstrated in two steps. First, we bound bio- λ DNA to magnetic beads coated with streptavidin, washed off unbound molecules, and digested the bound DNA with *StuI*. The fragments in the supernatant were separated on a 0.8% agarose gel (Fig 5). *StuI* releases a 12 434 bp fragment from the left end (band 2) and a 7888 bp piece from the right (band 3). The first and fourth bands, 19 044 bp and 6995 bp, respectively, come from within the molecule and can be used as size standards. Three smaller fragments are not shown.

Figure 5a compares the *StuI* fragments of bio- λ DNA (lane 1 and 2), and unmodified λ DNA (lane 3 and 4). Both samples were run before (lanes 1,4) and after binding (lanes 2,3) to the beads. The bio- λ DNA is clearly missing the second band (lane 2), which corresponds to the left end. The right end (band 3) was not affected. A small amount of DNA in band 2, approximately 5%, may originate from 3' biotinylated nicks distributed homogeneously over the λ DNA. This would lead to a constant background in each band. The control experiment with non-biotinylated λ DNA (lane 3) shows that the effects of nonspecific binding can be ignored.

Figure 6. Biotinylated λ monomers and multimers are specifically bound to the functionalized Au surface (left half). In contrast, very little DNA is bound to the glass surface (right). Applying an electric field stretches the molecules from the Au edge. The scale bar is 16 μm , about the size of a λ monomer. The molecules were embedded in 0.8% agarose in TBE to prevent convectional flow, and the pinhole of the confocal microscope was 70% open.

Figure 5b shows the *StuI* digested fragments of bio- λ -dig DNA before (lane 1) and after (lane 2) binding to streptavidin beads. Once again, the intensity of the second band (left end) is clearly reduced. By adding anti-digoxigenin antibodies to the same supernatant (final concentration, 0.3 μM), and incubating the sample for 1 h at room temperature (lane 3), band 3 (the right end) is strongly reduced. The antibody bound to the digoxigenin retards the fragment. It cannot be seen as a sharp band, possibly because of the continuous release of DNA from the antibody during the gel run. The specific reduction of the right end by anti-digoxigenin verifies the incorporation of dig–dUTP to the right end only.

The left and right ends of λ DNA (and its multimers) can therefore be modified with different labels simply by filling them appropriately. By measuring the intensity profile of lane 3 we determined that about 15% of the right end runs at the normal location. This value is 10% higher than the one found in the bio- λ DNA experiments (Fig. 5a). The difference in background between single and double end labeling may be caused by the extended reaction time in the preparation of double labeled ends. This treatment may have caused additional nicks, which were labeled in the second polymerase reaction.

Microscopy of λ DNA tethered to a Au surface

Once the DNA molecules are bound to the Au surface they can be imaged by fluorescence microscopy. We used confocal microscopy for the following reasons. (1) Fluorescence is detected within a very restricted focal plane. As a result background fluorescence is considerably reduced. (2) Scanning laser illumination in combination with light sensitive photo multipliers as detectors reduces the total exposure of the samples, and this reduces fading of the stained DNA molecules. (3) Individual DNA molecules can be imaged within zoomed areas as small as 50 μm^2 without bleaching the vicinity.

For the imaging experiments, we used a mixture of monomers and 100–250 kbp multimers, as determined by pulsed field electrophoresis (results not shown). We first stained the samples

Figure 7. Bio- λ monomers and multimers were bound to the Au surface and stretched by viscous flow. Images were taped on video during microscopy in order to optimize the time resolution. Pictures were then digitized and contrast enhanced. They show random coiled DNA (a), and DNA stretched by the flow (b). During flow reversal (c), DNA rotates 180° (see arrows). The time elapsed between each image in (c) is 2 s. The flow forces can be shown to be about 11 pN (see text).

with YOPRO1 and tethered them to the functionalized Au surface as described above. Figure 6 shows the edge of a 2 mm wide Au line. Part of the Au surface covers the left side of the picture. In contrast to the glass surface (right side), all DNA molecules are specifically bound to the Au. The DNA molecules on the Au edge are stretched out in an electric field of 6 V/cm.

To demonstrate specific binding of the biotinylated left end, we stretched tagged bio- λ DNA using a laminar flow of buffer solution. The flow was introduced by adding a drop of buffer to one side of the chamber. The buffer was then sucked into the chamber by capillary forces, initiating the flow. The flow speed was reduced by increasing the viscosity of the TE buffer with 56% sucrose. Figure 7a shows tethered DNA as a random coil, just before adding buffer. Flow, induced from the right side, stretched and oriented the molecules within seconds towards the left (Fig. 7b). Switching the flow direction reversed the orientation of the DNA. Figure 7c shows the rotation of a single molecule around its bound end (see arrows) as a function of time. We repeated this experiment several times without stripping the DNA from the surface. A 2.6-fold higher viscosity (62% sucrose) shears off most of the DNA molecules during the preparation of the sample.

The above experiments demonstrate that λ DNA multimers can be tethered specifically by their left end. The width of the Au, however, introduces considerable ambiguity into the location of the end, which we have overcome by exploiting a second advantage of Au, namely that it can be deposited on surfaces in micro structures. By anchoring DNA molecules to a single Au line, a fraction of the DNA contour length can be used to stretch DNA molecules from a well-defined point. In Fig. 8,

Figure 8. Bio- λ multimers tethered from a micro-fabricated Au line 5 μ m wide and aligned in an electric field. DNA was embedded in 0.8% agarose in TBE, and the electric field was 5 V/cm. Images A (scale bar = 80 μ m) and B (scale bar = 16 μ m) are different magnifications. The relatively low contrast of these pictures is because a low laser excitation intensity was used to prevent fading and photo chemistry, since no antifading agent was added in this experiment.

molecules were anchored to a functionalized Au line 5 μ m wide and stretched in an electric field of 5 V/cm. Figure 8a shows aligned λ DNA multimers at low magnification. Figure 8b is a zoom into a region with 5 DNA molecules of increasing length ($11.8 \pm 2.5 \mu$ m to $52.9 \pm 2.5 \mu$ m). The smallest one is about the size of a monomer, the largest the size of a 4-mer. The scale bar shows the length of a λ monomer for comparison. In addition, there are unbound DNA molecules free in solution.

DISCUSSION

We have shown that reduced biotin-HPDP can be used as a reagent to build up a layer of biotin-HP thiol on vapor-deposited Au surfaces. Our data give the following insight into the structure of the resulting layer: The high reactivity of biotin-HP thiol is shown by the fast coverage of the surface. This result agrees with Buck *et al.*, who used different alkane thiols (29). The final coverage of the layer is about 98% of the accessible surface area. The functionalized surface has a streptavidin binding capacity of 12 ± 1 pmole/cm², about 24% of the surface. We assume

that the small pyridine thiols are interspersed with the long chain biotin-HP thiols, and this may make the biotin group more accessible, consequently increasing the binding capacity [see Häussling *et al.* (21)]. We conclude from the competition experiments (Table 1) that the treatment of Au surfaces with reduced biotin-HPDP leads to very specific streptavidin binding.

We have also worked out a method for specifically labeling both ends of λ DNA by filling the protruding strands with dNTPs and modified dUTP. Under our conditions, different modified dUTPs can be incorporated at each end. This may be more generally useful than the use of oligonucleotides complementary to each end (10), although the fact that nicks are also labeled, may restrict this approach to smaller molecules.

By applying both methods, it is possible to bind biotin labeled λ DNA by the left end to a Au substrate functionalized with biotin and activated with streptavidin. The tethered λ DNA multimers can then be stretched by hydrodynamic forces and imaged. Figure 7 shows the free rotation of λ DNA induced by changing flow direction. We conclude that the stretched DNA does not adhere to the surface and must therefore be exposed to a non-zero flow velocity. This observation allows us to calculate the forces applied to the streptavidin-biotin-thiol anchor by fluid flow. Stretched DNA can be seen as a long rod (radius a , length L) in a viscous flow (viscosity η). The friction force F is (30):

$$F = 6\pi\eta c\nu, c = \left(\frac{3a^2L}{4}\right)^{1/2} \frac{(La/2)^{3/2}}{(3/2)^{1/2} \{2\ln(L/a) - 0.11\}}$$

Using the size of λ DNA, the viscosity η of a 56% sucrose solution (32 cP) (31), and the flow velocity $\nu = 60 \mu\text{m/s}$, the force applied to the anchor is 11 pN. The flow velocity near the stretched DNA was measured by simultaneously determining the velocity of unbound DNA molecules floating in the same focal plane. The high velocity gradient near the surface, however, and the fact that free molecules move with a velocity averaged within their diameter, gives a relative error in ν of about 27% or ± 3 pN (assuming a parabolic velocity profile between two parallel plates 80 μm apart). The anchor may resist higher forces, but because the DNA was removed from the surface during sample preparation at higher sucrose concentrations, we were unable to determine the upper limits.

We further showed that molecules of 50–200 kbp can be aligned on micro fabricated Au lines in an electric field of 5 V/cm. Using an electric field for stretching DNA molecules applies a much weaker force than a flow field. For example, the electrostatic force $F_{el} = qE$ applied in an electric field of $E = 6 \text{ V/cm}$ on λ DNA monomers is $\sim 5 \times 10^{-13} \text{ N}$, assuming a charge density on the DNA of 0.1 e per base pair (32). If the same electric field were applied to a DNA molecule of 1 Mbp, the resulting force ($F_{el} \sim 10 \text{ pN}$) would be of the same order of magnitude as we calculated for shear oriented molecules. This suggests that our anchoring mechanism can be used for stretching DNA of at least this size.

This aligning technique can thus be used to stretch out very long molecules indeed. One application of this technique might be to improve the imaging of chromosome length DNA molecules. A possible combination of this aligning technique with *in situ* hybridization might be used to localize with high accuracy genes within chromosomal length molecules. The anchor can also resist forces typically applied in SPM techniques. Thus a scaling down of this technique by anchoring DNA to nanostructures might be a way to align DNA for future sequencing methods using SPM technology.

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