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A single-step competitive binding assay for mapping of single DNA molecules

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ABSTRACT

Optical mapping of genomic DNA is of relevance for a plethora of applications such as scaffolding for sequencing and detection of structural variations as well as identification of pathogens like bacteria and viruses. For future clinical applications it is desirable to have a fast and robust mapping method based on as few steps as possible. We here demonstrate a *single-step* method to obtain a DNA barcode that is directly visualized using nanofluidic devices and fluorescence microscopy. Using a mixture of YOYO-1, a bright DNA dye, and netropsin, a natural antibiotic with very high AT specificity, we obtain a DNA map with a fluorescence intensity profile along the DNA that reflects the underlying sequence. The netropsin binds to AT-tetrads and blocks these binding sites from YOYO-1 binding which results in lower fluorescence intensity from AT-rich regions of the DNA. We thus obtain a DNA barcode that is dark in AT-rich regions and bright in GC-rich regions with kilobasepair resolution. We demonstrate the versatility of the method by obtaining a barcode on DNA from the phage T4 that captures its circular permutation and agrees well with its known sequence.

1. Introduction

While base-by-base sequencing provides genomic information with the ultimate resolution of single nucleotides it suffers from several limitations. Traditional Sanger shotgun sequencing protocols, as well as its more cost- and time-effective followers, are all based on fragmenting the unknown DNA and sequencing each fragment independently [1]. The results from each fragment are subsequently pieced together using powerful bioinformatics algorithms. However, long-range information is lost in the process and repeated regions are notoriously difficult to sequence reliably.

Optical mapping techniques on the other hand are based on direct imaging of intact single DNA molecules where the internal order is preserved, however at the price of a reduced resolution set by the optical system and the packing density of the DNA. Applications include mapping of structural variations, scaffolding for sequencing and identification of pathogens like viruses and bacteria. The earliest forms of mapping were banding of condensed chromosomes giving a resolution on the order of 1–10 Mb. Mapping based on fluorescence *in situ* hybridization (FISH) gives more specific information but with similar resolution [2]. On the other hand, stretching the DNA on surfaces [3–5] or in nanochannels [6] and visualizing it using fluorescence microscopy has attracted significant attention with its relative ease of use and higher resolution. Using standard fluorescence microscopy techniques, where DNA is stretched in nanochannels or on glass, a resolution of 1 kbp is obtainable.

There is a fair range of optical methods, mainly based on fluorescence microscopy, for large-scale genome mapping on the single DNA molecule level [7–10]. Early methods include DNA immobilization on patterned surfaces, fluorescent labeling, and cutting with restriction enzymes, in order to detect the specific sites the enzyme recognizes and thereby create a map of the DNA [10]. Similar approaches, using nicking enzymes, where the DNA remains in one piece have also been demonstrated [8]. More recently, Neely et al. demonstrated how methyltransferases can be used to attach fluorophores to specific 6-base sequences on DNA [11]. Using several different methyltransferases a multicolor high-resolution mapping can be obtained in an efficient way.

Using nanofluidic channels to linearize long DNA molecules, rather than stretching them on glass slides, has the benefit of ensuring reproducibility. When a large piece of DNA is introduced into a nanochannel, the confinement will lead to spontaneous

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stretching of the DNA along the channel, with an extension that scales linearly with contour length. The DNA is thus held in place without the need of any external stretching forces, so that DNA of arbitrary length can be analyzed. Previously, Reisner et al. have demonstrated the use of nanofluidic channels in combination with local DNA melting to obtain a DNA barcode [12]. By using a DNA dye that has a much brighter fluorescence when bound to double stranded DNA than to single stranded they obtained a fluorescence intensity profile along the DNA that reflects the underlying AT/GC ratio. In order to detect melting at an experimentally convenient temperature a denaturant, formamide, is added to decrease the strength of the hydrogen bonds. While melting mapping is capable of producing high-quality barcodes, there are two important experimental difficulties that may deter a wider audience. Firstly, it requires a detailed control of the temperature in the nanochannels, which is experimentally tricky and in turn makes the setup more complicated. Secondly, it requires a noxious chemical, formamide, which acts as a denaturant. Formamide also hampers the imaging conditions in the microscope by reducing the fluorescence intensity of the dye. An optimal future mapping method based on the same simple underlying principle – a barcode based on differences in fluorescence intensity between AT-rich and GC-rich regions – would thus be one where the pattern is formed *outside* the chip, and where the measurements can be performed at equilibrium and at ambient temperature without the use of any toxic reagents. For simplicity and ease of use it would be desirable that the sample preparation can be done in a *single step*.

An alternative mapping assay that fulfills these criteria would be to use DNA-binding molecules with different sequence specificities and dissimilar fluorescent properties. One possible approach would be to use sequence specific fluorescent dyes. However, existing dyes typically have insufficient sequence specificity or too weak fluorescence for detection in a standard fluorescence microscope. Instead, we combine a non-fluorescent molecule with exceptional sequence specificity with a commonly used fluorescent DNA dye.

We here demonstrate an assay that can be used to obtain barcodes in a simple *single-step* procedure by adding a mixture of netropsin, a natural antibiotic with a very high specificity for AT-rich sequences [13,14] and the bis-intercalating dye YOYO-1 to a long piece of DNA that is stretched in a nanofluidic channel. The result is a reproducible fluorescence intensity profile along the DNA that reflects the underlying AT/GC content on the single DNA molecule level.

2. Materials and methods

2.1. Chemicals

The TBE buffer was purchased from Medicago as 10× TBE tablets and dissolved in milli-Q water to the desired concentration. β-Mercaptoethanol (Sigma–Aldrich) was added to the buffer (3% v/v) to suppress photonicking of the DNA. Netropsin was purchased from Sigma–Aldrich and YOYO-1 was purchased from Invitrogen and subsequently added at the desired concentrations by dilution in buffer. Throughout the study the term "excess" relates to the ratio between the concentrations of netropsin and YOYO-1. DNA from phage lambda (λ-DNA, 48.5 kbp) was purchased from New England Biolabs and T4GT7 DNA (T4-DNA, 166.5 kbp) provided by Nippon Gene and purchased through Wako.

2.2. Stretching DNA on glass slides

For stretching on glass slides, λ-DNA was stained with YOYO-1 (one dye per 10 basepairs) and heated for 3 h at 50 °C. Netropsin was subsequently added and the DNA was stretched on positively charged glass slides obtained from Thermo Scientific (Menzel-Gläser). The experiments were performed in 2× TBE buffer with λ-DNA stained with YOYO-1 (one dye per 10 basepairs) at 8000 times excess of netropsin (in relation to YOYO-1).

2.3. Stretching DNA in nanofluidic chips

The nanofluidic chips were fabricated in fused silica using methods described elsewhere [6]. The channels were approximately $100 \times 150\ nm^2$ in cross-section and 500 μm long. The different DNAs were either prestained with YOYO-1 (one dye per 10 basepairs) after which netropsin was added, or netropsin and YOYO-1 were premixed and added to the DNA. The experiments were performed in 0.5x TBE buffer.

The DNA is loaded into one of the microchannels of the chip and transferred to the nanochannel array by an applied pressure difference. By applying pressure over two connected microchannels the DNA is subsequently injected into the nanochannels.

The microscope used is a Zeiss Axiovision equipped with a Photometrics Evolve EMCCD camera and a 100× oil immersion objective (NA = 1.46) from Zeiss.

Image stacks with 100 images were recorded for each molecule with an exposure time of 100 ms per image at 10 frames per second.

2.4. Analysis

Data analysis was performed with the freeware ImageJ (http://rsbweb.nih.gov/ij/) and a custom-written MatLab based software. For the studies on glass slides the traces were obtained from a single image and a profile plot was obtained using ImageJ. For DNA confined in nanochannels a kymograph (timetrace) was extracted for each movie using ImageJ and aligned using the MatLab software. The initial alignment is done on the center of the molecule to eliminate the effects of drift along the nanochannel. The intensity profile from the molecule is fitted to a linear combination of error functions, an extension of the procedure used in Ref. [15].

A finer alignment procedure is subsequently performed on distinct features (peaks or dips in intensity) along the DNA. This alignment step is commonly performed in multiple steps where the initial steps are performed on a Gaussian or average smoothed kymograph to suppress the effects from noise. This alignment step is based on finding local maxima/minima in the profile for each frame and then match the closest features between neighboring frames. The result is then verified by manual inspection. The actual alignment between two features is done by linearly extracting/compressing the profile between the adjacent tracked features for all frames to match the average distance between the features in all frames (lines in the kymograph).

2.5. Theoretical barcodes

The theoretical barcodes for λ-DNA and T4GT7-DNA were calculated by first running a moving average with a window size of five nucleotides over the whole sequence (Accession Nos. NC001416 for λ-DNA and NC000866 for T4 DNA and taking into account that nucleotides number 165,255–168,510 are lacking in T4GT7) to obtain a measure for the relative binding affinity of the ligand which binds to four consecutive basepairs in the minor groove. To make a direct comparison between the theoretical barcodes and the experimental data possible, the resolution of the experimental microscope setup is accounted for in the theoretical map by convolution with the point-spread function (PSF) of the microscope. The PSF was estimated by fitting a Gaussian profile to the intensity profile of a quantum dot imaged using the same microscope. A standard deviation of approximately 0.3 μm was obtained.