Published in final edited form as:

Anal Chem. 2011 November 1; 83(21): 8073–8077. doi:10.1021/ac202506j.

Single DNA Molecule Patterning for High-Throughput Epigenetic Mapping

Aline Cerf¹, Benjamin R. Cipriany², Jaime J. Benítez¹, and Harold G. Craighead^{1,*}

¹School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853, United States

²Department of Electrical and Computer Engineering, Cornell University, Ithaca, New York 14853, United States

Abstract

We present a method for profiling the 5-methyl cytosine distribution on single DNA molecules. Our method combines soft-lithography and molecular elongation to form ordered arrays of more than 250,000 individual DNA molecules immobilized on a solid substrate. The methylation state of the DNA is detected and mapped by binding of fluorescently labeled methyl-CpG binding domain peptides to the elongated dsDNA molecules and imaging of their distribution. The stretched molecules are fixed in their extended configuration by adsorption onto the substrate so analysis can be performed with high spatial resolution and signal averaging. We further prove this technique allows imaging of DNA molecules with different methylation states.

Epigenetics is the study of modifications in gene expression caused by chemical modification of the chromosome with no changes in the underlying DNA sequence. Among the numerous chemical modifications, DNA 5-cytosine modification, or methylation, is one of the most widely studied mechanisms influencing gene regulation. One of the effects of DNA methylation is to physically impede the binding of transcription factors to their recognition sites, and another is to bind methyl-CpG-binding domain (MBD) proteins involved in the modification of chromatin. In recent years, the field of epigenetics has become one of the most rapidly growing branches of molecular biology with increasing effort devoted to efficiently characterizing the human epigenome. The number of diseases suspected of being influenced by DNA methylation is growing and includes Alzheimer's disease, schizophrenia, diabetes, atherosclerosis, Parkinson's disease, cancer, among others¹. Interrogation of DNA methylation patterns in regulatory regions such as CpG islands has become an important tool for medical diagnostics and understanding of gene regulation. DNA methylation detection could serve, in particular, as a tool in cancer diagnosis and monitoring of treatment.

Current methods for methylation assessment and analysis include sequencing after bisulfate conversion², methylation-specific polymerase chain reaction (PCR)³, methylation-sensitive single-nucleotide primer extension (Ms-SNuPE)⁴, combined bisulfate restriction analysis

*To whom correspondence should be addressed. hgc1@cornell.edu; Fax: (607) 255-7658.

(COBRA)⁵, methylated DNA immunoprecipitation⁶, restriction landmark genome scanning⁷, hybridization arrays^{8–9}, among others. Efficient individual molecule analysis could reveal information lost in current ensemble methods of bulk material analysis and allow investigation of single cells. While the benefits of single molecule analyses are known and research is underway on new such methods, they are not yet available for general use. Current single molecule methods being studied include fluorescent detection of methylation in nanofluidic systems^{10–11}, and observation of rate dependence of polymerase activity as influenced by methylation¹². However, none of these methods is yet in general use in epigenetic analyses and the need for robust methods still exists.

In this correspondence, we present a method for transferring regular arrays of individual elongated DNA molecules onto SiO₂ substrates for optical analysis. In this method, the DNA molecules are labeled with fluorescent MBD1 probes to reveal the methylation sites. As a test of our technique, we used *in vitro* methylated phage-lambda DNA molecules as a model system. In order to achieve single-gene resolution, DNA is elongated by capillary forces and organized on a topographically-patterned substrate. The elongated molecules are arrested in their extended configuration by transfer-printing on a surface for subsequent high-throughput optical analysis. We show that the spatial location of methylated sites within the molecules can easily be imaged and mapped. Our approach is also consistent with emerging techniques capable of optically mapping the lengths of enzymatically cleaved DNA fragments.

EXPERIMENTAL SECTION

Preparation of biological materials

Core members of the MBD protein family (MeCP2, MBD1, MBD2, and MBD4) share a methyl-CpG-binding domain that has a specific affinity for methylated CpG sites in double-stranded DNA. Here, to assess global levels of DNA methylation, we used MBD1 peptide as our probe, which has been shown to preferentially bind a symmetrically methylated CpG motif¹³. The phage lambda DNA solution (Sigma, 48 502 bp, 329 µg/ml) was heated at 65°C for 5 min and dipped into ice water to avoid molecular concatenation. Methylation of lambda DNA (Sigma Aldrich) was performed using CpG methyltransferase (*M.SssI*) from New England Biolabs (NEB) according to their standard protocol. *SssI* can methylate all 3113 CpGs in the 48.5 kbp genome which are evenly distributed throughout the entire molecule (approximately one CpG island every 16 basepairs). Methylation state was controlled with a methylation-sensitive cleavage of DNA (*MspI*, NEB), and verified by an electrophoresis gel.

MBD1 peptide labeling

MBD1 peptide was purchased from Abcam (Ab4918). Peptides were labeled with Alexa Fluor® 488 carboxylic acid, TFP ester (A-10235, Invitrogen), which targets primary amines. The buffer pH was kept close to neutral (pH 7.4) to achieve more specific labeling of the amine terminus. Labeled peptides were purified by gel filtration (Superdex Peptide 10/300 GL, GE Healthcare, 17-5176-01) using an ÄKTA FPLC system (GE Healthcare). The Alexa Fluor 488 labeled MBD1 retained its specificity for methylated DNA.

Methylated and non-methylated lambda DNA (Sigma Aldrich) were incubated against Alexa488 MBD1 to determine the specificity of the peptide against the methylated binding sites. Methylated and non-methylated lambda DNA were diluted in 10 mM Tris-HCl / 1 mM EDTA, pH 8, to 100 µg/mL. One µg each were incubated in molar excess with a two-fold concentration of Alexa488-labeled MBD1 relative to the concentration of CpG sites in the DNA. Incubation was conducted for 2 hours in the dark, at room temperature. Each solution was then counterstained with BOBO-3 intercalating dye (Invitrogen) at a 1:5 dye per base pair ratio during 2 hours in the dark at room temperature. Finally, equal volume of formalin with 0.1% v/v Triton- X100 was added and cross linking was conducted for 2 minutes.

Chemical functionalization of glass substrates

The glass cover slips were coated with (3- Aminopropyl)triethoxysilane (APTES; Sigma Aldrich 440140) molecules. The chemical functionalization was carried out by immersion of the substrates into a freshly prepared solution containing APTES molecules diluted at 1% with ethanol for 15 min; the slides were then rinsed with ethanol, dried under nitrogen stream, and heated on a 140°C plate.

Operational procedure

To direct the capillary assembly of phage lambda DNA, we used polydimethylsiloxane (PDMS) stamps with topographical cavities obtained from the replication of a positive silicon master. The silicon micropatterned master was achieved by ultraviolet photolithography and the pattern transfer by deep reactive ion etching. The PDMS prepolymer solution containing a mixture of 10:1 mass ratio of PDMS oligomers and a reticular agent from Sylgard 184 Kit (Dow Corning, Wilmington, DE) was then poured onto the silicon master and cured at a temperature of 80°C during 2 hours. The cured PDMS was peeled off and cut into 1.8 cm × 1.4 cm stamps. In a general manner, the design of the topographic patterns requires a prior reflexion in terms of distribution, dimension, depth, and orientation. For DNA molecules' assembly, the silicon master was designed with protruding microfeatures 5 µm and 8 µm in diameter, 5 µm high and with different periodicities (20 µm, and 25 µm). Therefore, the corresponding PDMS stamps are the negatives of the master and consist of microcavities with the same sizes. The directed assembly is carried out using a dedicated setup. The microstructured PDMS stamp where we want the DNA molecules to be assembled is placed on a motorized translation stage below a fixed glass spreader at a distance of about 1 mm. The experiment is conducted at ambient temperature. A 15 µl droplet of the final DNA molecules' solution is injected between the glass and the substrate. The liquid contact line is then moved over the substrate at a constant velocity of 0.5 mm/s for the trapped DNA molecules to be stretched. The assembly is performed throughout the entire surface of the PDMS stamp, so approximately over an area of more than 1 cm². To transfer the formed DNA arrays, the PDMS stamp with the assembled DNA molecules is then brought into contact with a (APTES)-coated cover slip for 2–3 min. The PDMS stamp is then peeled away (Figure 1). The molecules' transfer was controlled under an inverted epifluorescence microscope (100X oil immersion objective) from Olympus coupled to a 512×512 CCD camera (Photometrics). The samples were imaged under illumination at 475 nm and 560 nm accordingly with no cross-excitation detectable at 535 nm nor 645 nm. Green and red images were combined using ImageJ software.

RESULTS AND DISCUSSION

In the directed assembly technique, patterning is used to create a well-defined spatial distribution of forces that direct the motion of molecules in solution towards specific areas of a substrate. In our case, we use a PDMS stamp with topographical features to direct that assembly process. The experimental parameters, namely, the sample concentration and the displacement speed have been adjusted to enable the trapping and stretching of individual DNA molecules with high placement accuracy as previously reported¹⁴. After capillary assembly, the resulting DNA array is immediately transferred onto the APTES-coated cover slip (Figure 1). In general, for the transfer to occur, the molecules need to have more affinity for the target substrate than for the PDMS stamp's surface. In the present case, the target substrate is hydrophilic (PDMS vs APTES) with a measured contact angle of $108^{\circ} \pm 2^{\circ}$ and $61^{\circ} \pm 2^{\circ}$ respectively so the transfer is performed reliably. We obtain regular arrays of single phage lambda DNA molecules adsorbed on APTES-coated substrates following a Poisson distribution and with a 91% coverage rate¹⁵. First, as a control experiment, we incubated non-methylated BOBO-3 stained lambda DNA against Alexa488-labeled MBD1 peptides. As MBD1s are CpG-pattern-specific, no binding should occur. Figure 2 shows the results after capillary assembly and transfer. If we consider the first row of panels Figure 2, at 560 nm (Fig. 2a), the fluorescence micrograph shows the presence of a regular array of individual BOBO-3 stained DNA molecules.

We notice that DNA molecules are homogeneous in length $10.37 \mu\text{m} \pm 1.60 \mu\text{m}$ and orientation. We observe in Figure 2b that at 475nm, no binding of MBD1 peptides is perceivable as expected. Consequently, the resulting overlay Fig. 2c is the equivalent of the fluorescence micrograph at 560 nm as there is no contribution from the image at 475 nm. Now, the second row Figure 2 shows the experiment of interest where *in vitro* methylated lambda DNA molecules are incubated against Alexa488-labeled MBD1 peptides, assembled and transferred onto an APTES-coated cover slip. We observe that this time, contrary to the first row Figure 2, the DNA array is visible at 560 nm but also at 475 nm. The overlay Figure 2f of the images taken at 560 nm (Fig. 2d) and at 475 nm (Fig. 2e), shows high-degree co-localization of MBD1 to methylated DNA. This suggests homogeneous MBD1 binding all along the individual molecules as predicted by the dense methylation pattern of the methylated DNA substrates (one CpG island every 16 basepairs so non-methylated domains are imperceptible). This proves that MBD1 binds specifically to methylated DNA and that the spatial location of binding sites within the molecule can easily be mapped. Here again we determined the length of the molecules based on the images taken at 560 nm (BOBO-3 nucleic acid stained). We observe that MBD1-bound methylated lambda DNA consistently stretches to a length of $10.56 \mu\text{m} \pm 1.16 \mu\text{m}$, same as nonmethylated lambda DNA molecules. Regardless of their methylation state, molecules undergo the same stretching factor as length distributions from methylated and non-methylated DNA are both centered around $10 \mu\text{m}$ (see supplementary information). Thus the stretching of lambda DNA molecules seems to be independent from the methylation state or pattern. We do not observe any shortening or contraction upon MBD binding unlike previous report¹¹. We do observe the length distribution is slightly broader in the case of non-methylated DNA but we attribute this to a sampling effect rather than an influence of MBD1 binding onto the

assembly mechanism. Third row Figure 2 shows the resulting DNA array obtained when mixing in bulk methylated and non-methylated DNA at 1:1 ratio along with Alexa488-labeled MBD1 peptides. We observe that some molecules are only visible at 560 nm (Fig. 2g) and others are visible in the two panels (at 560 nm (Fig. 2g) and at 475 nm (Fig. 2h)), implying that Alexa488-labeled MBD1 peptides have only bound to some of the DNA molecules, revealing the methylated DNA population from the non-methylated population. Figure 2i, the overlay shows equal proportion of red-colored molecules and yellow-colored molecules, resulting from green and red co-localization in the false-colored image overlay. Since both methylated and unmethylated molecules are elongated, linearized and aligned the same way, this suggests that the assembly mechanism is independent of methylation state and solution complexity.

Our technique allows elongating and organizing individual molecules on a solid support. One of its major advantages is that the labeling of the molecules with specific antibodies or enzymes can be performed either in bulk or directly on the surface after transfer. If the experimenter chooses to perform it in bulk, as the capillary assembly is conducted at high speed on a highly hydrophobic substrate (PDMS stamp), only the molecules are physically trapped and elongated, while the rest of the solution is dragged over the substrate without being deposited. Since assembly only occurs on the long-chain polymer strands of DNA, this method inherently removes free-dye or other contaminants. We obtain, as a result, regular arrays of individual molecules over more than one cm^2 in a reliable manner, so approximately two hundred and fifty thousand molecules over the entire substrate that are potentially analyzable. We have proven that we can image and map methylation upon binding of fluorescently-labeled MBD1s. The elongated molecules are arrested in their extended configuration by adsorption on the substrate so analysis or detection can be performed with high spatial resolution and signal averaging. From that perspective, there has been a growing interest in using methods to analyze single molecules with spatial resolution beyond the optical diffraction limit while benefiting from the advantages of established fluorescence spectroscopy techniques. As this methodology can be used onto various hydrophilic supports, it is compatible with all characterization methods including fluorescence microscopy (TIRF, PALM, STORM...), atomic force microscopy but also electron microscopy techniques. In fact, this technique is not only adaptable to parallel processing over various hydrophilic substrates but also hydrophobic substrates such as graphene. We have recently shown that we can transfer arrays of elongated DNA molecules to single-layer graphene¹⁶, opening the possibility for single-nucleotide resolution imaging of individual base-labeled DNA molecules or chromatin using transmission electron microscopy (TEM) and electron energy loss spectroscopy (EELS).

In summary, we demonstrate an affordable and fast technique to assess methylation patterns of CpG dinucleotides in single DNA molecules. By combining directed assembly on a PDMS stamp and microcontact printing, we benefit from the advantages of both techniques at the same time: the control over the assembly process and the flexibility and simplicity of the printing technique. This ability to controllably pattern large numbers of DNA molecules over large areas offers the opportunity for parallelized and high-throughput screening with high-resolution capabilities. In addition to research use, this simple and fast technique may prove to be especially useful for clinical studies as the procedure is straightforward and does

not require complex fabrication or preparation protocols. Further development will lead us onto high resolution optical imaging (PALM/STORM) to perform high-throughput genetic and epigenetic mapping of DNA and chromatin with sub-20nm resolution allowing multiple fluorescent labels to be spatially separated and resolved in the case of multilabeled DNA molecules or chromatin. This may permit molecular mapping and analysis with spatial resolution sufficient to identify multiple epigenetic marks on a single nucleosome or to distinguish marks on adjacent nucleosomes in native chromatin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work described was supported, in part, by the Cornell Center on the Microenvironment & Metastasis, a Physical Science Oncology Center supported by Award Number CA143876 from the National Cancer Institute and supported in part by National Institutes of Health grant DA025722. We thank Paul Solo way and his research group for very helpful discussions.

REFERENCES

1. Schumacher A, Petronis A. *Curr. Top. Microbiol. Immunol.* 2006; 310:81, 115. [PubMed: 16909908]
2. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE. *Nature.* 2008; 452:215. [PubMed: 18278030]
3. Fraga MF, Esteller M. *Biotechniques.* 2002; 33:632. [PubMed: 12238773]
4. Gonzalgo ML, Liang G. *Nat. Protoc.* 2007; 2:1931. [PubMed: 17703204]
5. Xiong Z, Laird PW. *Nucleic Acids Res.* 1997; 25:2532. [PubMed: 9171110]
6. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schübeler D. *Nat. Genet.* 2005; 37:853. [PubMed: 16007088]
7. Ando Y, Hayashizaki Y. *Nat. Protoc.* 2006; 1:2774. [PubMed: 17406534]
8. Bibikova M, Lin Z, Zhou L, Chudin E, Garcia EW, Wu B, Doucet D, Thomas NJ, Wang Y, Vollmer E, Goldmann T, Seifart C, Jiang W, Barker DL, Chee MS, Floros J, Fan J-B. *Genome Res.* 2006; 16:383. [PubMed: 16449502]
9. Nautiyal S, Carlton VEH, Lu Y, Ireland JS, Flaucher D, Moorhead M, Gray JW, Spellman P, Mindrinos M, Berg P, Faham M. *Proc. Natl. Acad. Sci.* 2010; 107:12587. [PubMed: 20616066]
10. Cipriany BR, Zhao R, Murphy PJ, Levy SL, Tan CP, Craighead HG, Soloway PD. *Anal. Chem.* 2010; 82:2480, 2487. [PubMed: 20184350]
11. Lim SF, Karpusenko A, Sakon JJ, Hook JA, Lamar TA, Riehn R. *Biomicrofluidics.* 2011; 5:034106.
12. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW. *Nat. Meth.* 2010; 7:461, 465.
13. Hendrich B, Bird A. *Mol. Cell. Biol.* 1998; 18:6538, 6547. [PubMed: 9774669]
14. Cerf A, Dollat X, Chalmeau J, Coutable A, Vieu C. *J. Mater. Res.* 2011; 26:336, 346.
15. Cerf, A. Directed assembly of nano-objects. Toulouse University; 2010.
16. Cerf A, Alava T, Barton RA, Craighead HG. *Nano Lett.* 2011

Figure 1.

Schematic representation of the experimental procedure to generate the DNA arrays for assessment of DNA methylation. 1), 2) and 3) represent the capillary assembly process. The liquid meniscus of solution containing MBD1 probes and methylated and/or unmethylated DNA molecules is dragged over a microstructured PDMS stamp. The molecules are physically trapped and elongated as the meniscus is displaced across the substrate. 4) and 5) represent transfer-printing of the DNA array on an APTES-coated cover slip. The PDMS stamp with the DNA array is contacted with the APTES-coated cover slip for two minutes and then peeled-away.

Figure 2. Assessment of DNA methylation using capillary assembly

Fluorescence micrographs of elongated methylated and/or non-methylated BOBO-3 labeled lambda-DNA molecules incubated against methylation-sensitive Alexa488-labeled MBD1 peptides. First column and second column correspond to fluorescence micrographs taken at 560 nm (emission at 645 nm) and at 475 nm (emission at 535 nm) respectively. The third column corresponds to the composite image resulting from the false color overlay of the first column (red) and the second column (green) micrograph for each case of figure. First row (panels 2a, 2b, 2c): Control experiment using non-methylated DNA molecules. Second row (panels 2d, 2e, 2f): Methylated DNA molecules. Third row (panels 2g, 2h, 2i): 1:1 mixture of methylated and non-methylated DNA molecules. For each row, the fluorescence micrographs were taken from the same area. Scale bars correspond to 4 μ m.