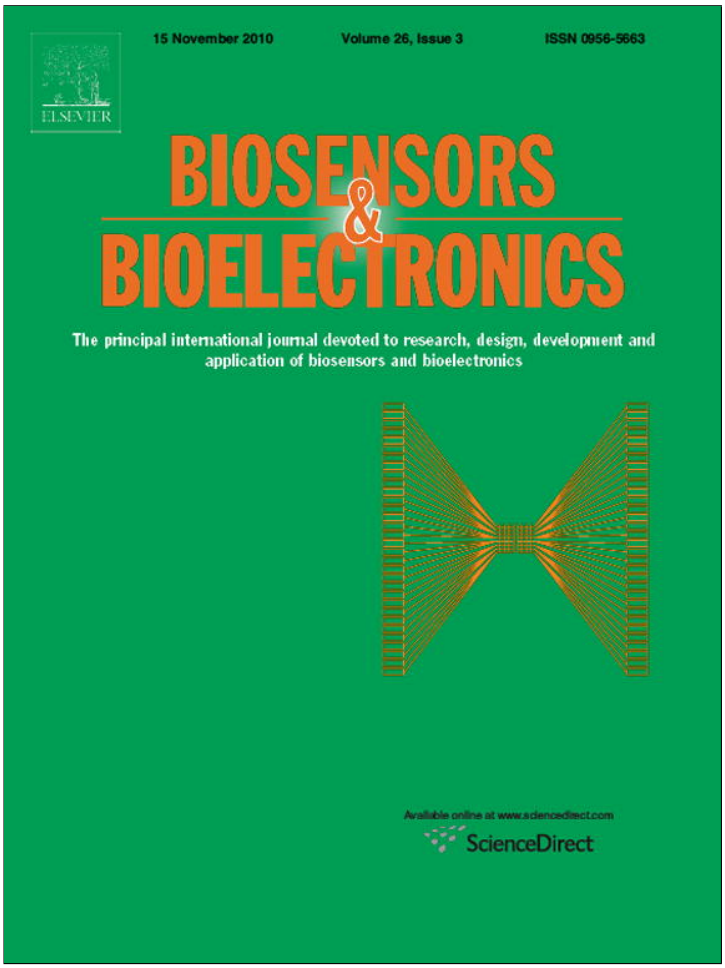

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# Label-free DNA methylation analysis using opto-fluidic ring resonators

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ABSTRACT

The opto-fluidic ring resonator (OFRR) is a sensitive label-free optical biosensor that is uniquely well suited for photonic and fluidic integration. For the first time we have explored the utility of this novel instrument for the analysis of methylation in oligonucleotides using the MBD-2 (methyl binding) protein as the capture molecule. This application has strong relevance to cancer research and future clinical tools through the study of methylation patterns in important gene promoters. In this work we quantitatively characterized the OFRR's response to artificially methylated ssDNA and dsDNA as a function of the number of methylated cytosines and DNA concentration. The effect of hemi- versus fully methylated oligonucleotides was also investigated. Additionally, anti 5-methylcytidine antibody was also used as the capture molecule and compared with MBD-2. It is found that the antibody has stronger affinity for ssDNA, whereas MBD-2 is much better at binding dsDNA.



## 1. Introduction

Genomic methylation analysis is of great importance for cancer research and clinics, since it enables earlier cancer diagnosis prior to the point of metastasis (Jones and Baylin, 2002). An ever increasing body of work within the field of epigenomics is strengthening the linkage between the hypermethylation of key nucleotide sequences and the advent of many different cancers (Jones and Baylin, 2002; Egger et al., 2004). As a natural component of cellular transcription, methyl groups are normally attached to select cytosine bases by methyltransferase enzymes in order to suppress expression of unnecessary proteins. Methylcytosines count for as much as 1% of all nucleotides in the human genome (Bird, 2002) and tend to be concentrated in CpG "islands" commonly seen in gene promoter regions (Baylin and Herman, 2000). However, abnormal methylation can prevent expression of important molecules, including those that govern cellular proliferation, which may lead to tumorgenesis (Kane et al., 1997; Herman et al., 1998; Baylin and Herman, 2000; Jones and Baylin, 2002).

In order to further study and predict this behavior, several methylation-specific assays have been developed (Herman et al., 1996; Kelsey et al., 1999; Fraga and Esteller, 2002; Gitan et al., 2002). Two of the most common assays are restriction enzyme digestion (Nelson et al., 1993) and bisulfite-conversion assays (Fraga and Esteller, 2002). The restriction enzyme process involves digesting a large segment of DNA into smaller segments. Methylated bases prevent the enzyme cleavage, and the difference between strand lengths is detected via southern blot or PCR amplification (Bruce et al., 2008). While sophisticated and reliable, this method can only focus on a few restriction sites at a time, and cannot be used to fully quantify the degree of methylation in a strand. The bisulfite method converts methylated cytosines to uracil bases that show up as thymine after PCR amplification and can be detected by sequencing or microarray hybridization (Gitan et al., 2002). Recently, a very interesting and sensitive FRET detection assay was developed based on bisulfate conversion (Feng et al., 2008). While it is popular and widely used, it is known to be somewhat unpredictable and inefficient due to degradation of the DNA sample during conversion (Bruce et al., 2008). A very new method, called single-molecule, real-time sequencing (SMRT) cleverly uses DNA polymerases to create high-throughput, dye-coded DNA strands (Flusberg et al., 2010).

A great deal of recent research has focused on utilizing the interaction between DNA and methyl binding proteins to gain a better understanding of methylation patterns and regulation (Hendrich and Bird, 1998; Wade, 2001). Methyl binding proteins are a family of transcription-regulating molecules, among which five are currently known, that actually bind directly to methylcytosines. This provides an advantage compared to the previously discussed methods because no pre-treatment or conversion of the sample

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DNA is required in order to detect it. While each protein shares the same binding region, their functions and behavior are decidedly unique from one another (Meehan et al., 1989; Hendrich and Bird, 1998). Only a few of these proteins, including MBD-2, are known to bind DNA in vitro. MBD-2 is also known to bind methylated DNA with very little sequence specificity. Therefore, it is possible to perform detection of the extent of methylation of any portion of the genome, making it very useful for cancer research and diagnostic applications. In addition to methyl binding proteins, methylcytosine-specific antibodies, such as anti 5-methylcytidine (anti-5mC), have also been employed in methylation studies. Various detection protocols, such as methylated-CpG island recovery assay (MIRA) (Rauch and Pfeifer, 2009) and methylated DNA immunoprecipitation (MeDIP) (Jacinto et al., 2008) have been developed based on those capture molecules. While these technologies can be very sensitive, they require fluorescent labeling, and therefore, have a limited ability to perform quantitative methylation analysis due to the signal bias resulting from the lack of precise control in molar ratio of fluorophores to analytes (Cox and Singer, 2004).

In contrast, label-free detection offers an excellent alternative, which directly and quantitatively detects the analyte without fluorescence labeling. However, to date very few label-free sensing formats have been employed in methylation studies. One such technique involves a non-optical nanowire-based field effect transistor (FET) sensor (Maki et al., 2008). While this platform offers exceedingly low limits of detection, the fabrication of nanowires is a delicate and costly process. Another recent label-free technique utilized surface plasmon resonance (SPR) to detect dsDNA using methyl binding proteins in a microarray format (Yu et al., 2010). SPR is a mature and sensitive assay, however the instrumentation is often quite costly.

Label-free detection can also be achieved using cylindrical optofluidic ring resonators (OFRRs), whose structure is illustrated in Fig. 1(a) (Suter et al., 2008). The OFRR is a thin-walled glass capillary. In the OFRR, the resonant light circulates repeatedly along the ring resonator surface and the resonance wavelength, $\lambda$, is determined by Gorodetsky and Ilchenko (1999):

$$\lambda = \frac{2\pi r n_{eff}}{m} \quad (1)$$

where $r$ is the ring's radius, $n_{eff}$ is the effective refractive index (RI) encountered by the circulating light, and $m$ is an integer multiple. The resonance wavelength shifts when the analytes are attached to the inner surface of the OFRR. In the past few years, detection of various biomolecules using the OFRR has been demonstrated, including virus (Zhu et al., 2008), DNA (Suter et al., 2008), protein (Zhu et al., 2007), and cancer biomarker in serum or blood (Zhu et al., 2009; Gohring et al., 2010).

The OFRR offers several advantages over other label-free sensors. First and foremost, it is among the most sensitive optical label-free biosensors to date. Refractive index sensitivities approaching 600 nm/RIU have recently been demonstrated while maintaining $Q$-factors greater than $10^5$, enabling RIU resolution below $10^{-7}$ and surface mass detection limit below 1 pg/mm$^2$, both of which are rarely achievable (Li and Fan, 2010). Second, the OFRR offers unparalleled opto-fluidic integration compared to other optical label-free sensors due to its capillary nature (White et al., 2007). Where other ring structures require independent integration of sample fluidics, the capillary serves as both fluidic channel and sensor head simultaneously, such that capillaries can be run in parallel to create a multiplexed panel for biomarker detection. Third, it potentially consumes very low sample volumes. While 100 μL of sample is usually used in our current experiments, the total sample consumption volume can eventually approach 1 μL or below, as the sample detection volume with the OFRR is only 1 nL (or less). We envision that the end users of the OFRR based sensors could be instrument manufacturers for clinical laboratories or clinical laboratories themselves. Even research laboratories could benefit a great deal from a label-free device of this nature, which can be made out of very low-cost components.

In the work described herein, DNA methylation analysis was demonstrated using a methyl binding protein, MBD-2, or antibody to capture methylated oligonucleotides to the OFRR inner surface, as illustrated by Fig. 1(b). Previous efforts have been published in conference proceedings (Suter et al., 2009a,b), however these included only very preliminary results and did not to provide a solid basis for comparison between the protein and antibody methods. The high specificity of MBD-2 proteins (or antibodies) and the quantitative, real-time optical sensing capabilities of the OFRR complement each other well. The sensor response to methylated oligonucleotides based on concentration and degree of methylation was investigated, showing that the protein can discriminate small changes in the degree of methylation, on the order of 3–5 methylcytosines. The MBD-2 protocol's preference for double stranded DNA (dsDNA) was also explored, and anti-5mC assays (shown in Fig. 1(c)) were carried out for comparison. It was observed that MBD-2 strongly prefers dsDNA, whereas the antibody binds more strongly to single stranded DNA (ssDNA), thus providing complementary approaches to methylation research.

## 2. Experimental

### 2.1. Materials

DNA samples (all 30 bases in length) were obtained from Integrated DNA Technologies (Coralville, IA). Details of the DNA sequences and methylation are given in supplementary material.

Recombinant MBD-2 protein was obtained from Bioclone Inc (San Diego, CA). Antibody selected was mouse $IgG_1$ raised against 5-methylcytidine, purchased from AbD Serotec (Raleigh, NC). Dimethyl adipimidate (DMA) and dimethyl pimelimidate (DMP) crosslinker was obtained from Pierce (Rockford, IL). Other reagents, including SSC buffer, PBS buffer, ethanol, hydrofluoric acid, 3-aminopropyltrimethoxysilane (3-APS), and protein G were obtained from Sigma–Aldrich (St. Louis, MO). OFRR capillary performs were purchased from Sutter Instruments (Novato, CA). Water, used as a diluent or in buffers, was purified to 18 MΩ using a Barnstead Easypure-UV system (Thermo Scientific, Asheville, NC).

### 2.2. Experimental configuration

The OFRR fabrication and characterization have been discussed previously (White et al., 2006; Fan et al., 2007). Briefly, a quartz glass capillary with an outer diameter of 1.2 mm and wall thickness of 150 μm was pulled under $CO_2$ laser illumination until the outer diameter was approximately 100 μm and the wall thickness was approximately 5 μm. Then hydrofluoric acid (HF) was passed through the capillary, etching the wall down even further. The OFRR was then placed on top of a thermal electric cooling (TEC) unit. The experiments were performed at room temperature. A syringe pump was used to push the fluid through the OFRR at a constant rate of 10 μL/min. Light from a tunable diode laser (1550 nm) scanned at 2 Hz across a spectral range of approximately 100 pm was coupled into the OFRR by an optical taper in contact with the OFRR (see Fig. 1(a)). The resonance wavelength emerged at the detector as a dip in the transmitted spectrum, which shifted in response to biomolecule attachment to the OFRR inner surface.

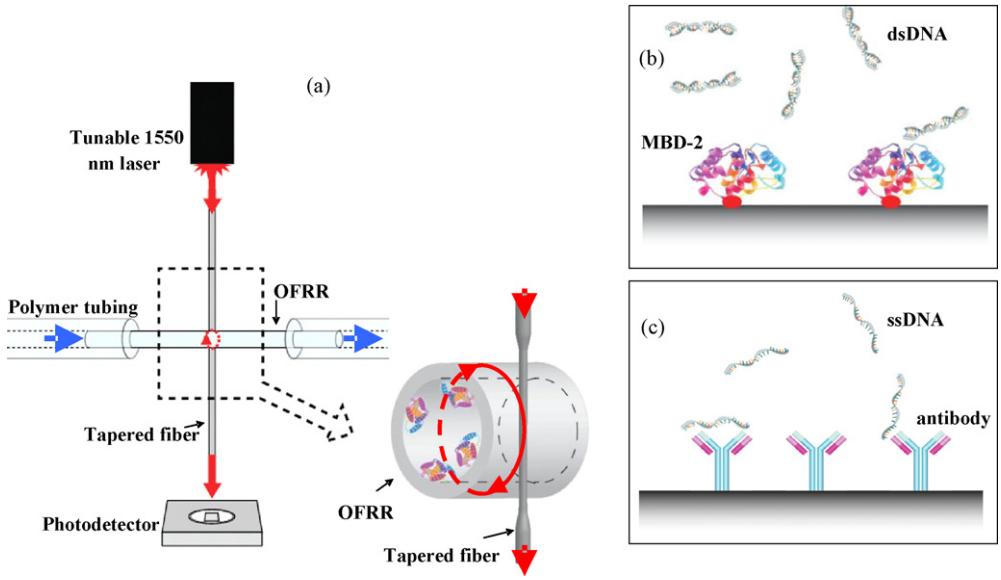


**Fig. 1.** (a) Experimental setup for OFRR. Inset shows biomolecule immobilization on the interior capillary wall. (b) Representation of methylated DNA strands binding to methyl binding proteins. (c) Representation of methylated DNA strands binding to immobilized antibodies inside the OFRR.

## 3. Results and discussion

### 3.1. Biomolecule immobilization

Based on our previous theoretical analysis, the resonance wavelength shift, $\delta\lambda$, is linearly dependent on the molecules attached to the OFRR inner surface (Zhu et al., 2007):

$$\frac{\delta\lambda}{S} \propto \sigma \tag{2}$$

where $S$ is the bulk RI change and $\sigma$ is the molecule surface density. In the following studies, we will use $\Delta n = \delta\lambda/S$ (in units of "refractive index units (RIU)") as the sensing signal.

To immobilize MBD-2 onto the inner surface of the OFRR, the OFRR was cleaned with HF and purged with air to generate a more hydrophobic surface. Then, 3-APS (1% by volume) was passed through and allowed to form a layer on top of this. To encourage crosslinking between silane molecules, the OFRR was then drained of fluid and heated with the TEC to 50 °C for 20–40 min. Then MBD-2 was crosslinked to the surface with 5 mg/mL DMA. After rinsing, the DNA sample was introduced. Fig. 2(a) shows a sample sensorgram representing the MBD-2 immobilization and sample DNA detection steps. Each step is represented as a large increase in sensing signal due to both specific and non-specific binding as well as bulk RI changes. The subsequent rinsing steps cause a substantial RI drop due to removal of non-specific binding and bulk RI change, but the resulting net change represents specifically bound analytes.

Fig. 2(b) demonstrates the sensor preparation process for anti-5mC based detection. The sensor was silanized the same way as in MBD-2, but then protein G (at 0.1 mg/mL) was covalently crosslinked to the 3-APS layer using DMP (at 5 mg/mL). Next, anti-5mC antibodies were introduced at a concentration of 10 μg/mL and allowed to bind to protein G. After this treatment, the capillary was ready to selectively immobilize methylated oligonucleotide targets. The inset of Fig. 2(b) shows the lowest demonstrated concentration of ssDNA captured with this method, which is 5 nM. The shift, or $\Delta n$ from this sample, $3.2 \times 10^{-5}$ RIU, is well above the RI detection limit of the sensor, which is closer to $2 \times 10^{-6}$ RIU based on the spectral noise observed from these sensorgrams, so it is theoretically possible to detect samples below 1 nM.

### 3.2. Methyl binding protein assay

Titration curves are plotted in Fig. 3 to compare the protein's performance in binding different types of DNA (fully methylated dsDNA and methylated ssDNA) at concentrations ranging from 1 nM to 10 μM with 5 methylcytosines each. An unmethylated dsDNA sample was used as a negative control. This data shows that MBD-2 binds much more strongly to dsDNA than to the other sam-

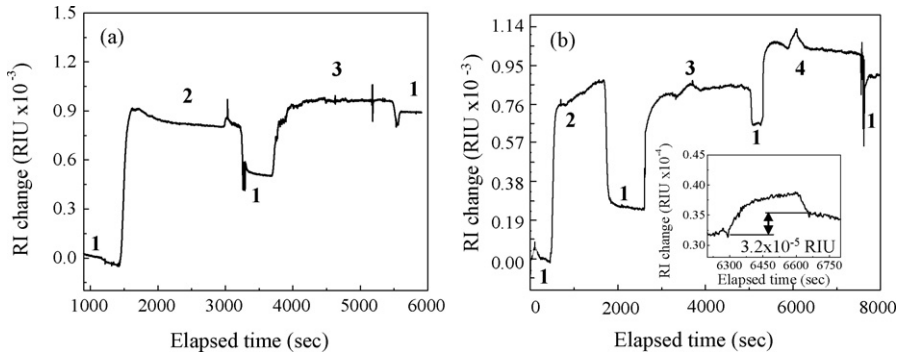


**Fig. 2.** Sensorgram showing DNA detection with MBD-2 (a) and anti-5mC (b). For (a) steps are: PBS buffer rinse **1**, MBD-2 protein immobilization **2**, and capture of 10 μM 30-mer dsDNA with 5 methylcytosines **3**. For (b) steps are: PBS buffer rinse **1**, protein G immobilization **2**, antibody immobilization **3**, and capture of 10 μM ssDNA with 5 methylcytosines **4**. Inset shows resolvable shift resulting from 5 nM methylated ssDNA.

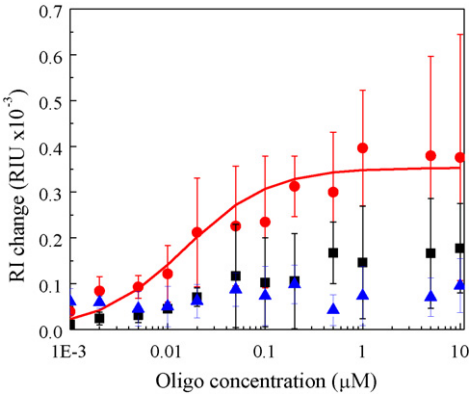


**Fig. 3.** Titration curves for MBD-2 experiments including 5-methyl dsDNA (circles), 5-methyl ssDNA (squares), and a negative control containing no methylcytosines (triangles). The fitted curve yields a $K_d$ value of 15 nM for dsDNA. The ssDNA data cannot be discriminated from the negative control.

ples, which is consistent with past studies of this protein (Hendrich and Bird, 1998). Compared to the negative control in Fig. 3, the ssDNA signal cannot really be statistically separated, indicative of weak interaction with the protein. Fitting a Langmuirian curve to the dsDNA data, we found an estimated dissociation constant (or $K_d$) equal to 15 nM. Although, to our knowledge, no data exists regarding the dissociation constant of MBD-2 specifically, our result is similar to that recorded for other methyl binding proteins (Valinluck et al., 2005).

The alternative protocol, using anti-5mC was also investigated based on binding curves for dsDNA and ssDNA. In Fig. 4(a) dsDNA was introduced to an antibody-covered sensor, showing a net RI change after dsDNA binding, which is almost 10 times smaller than the shift obtained from the MBD-2 method. Conversely, the antibody has a strong response to ssDNA samples with 5 methylcytosines, as shown in Fig. 4(b). Fig. 4(b) plots titration curves for ssDNA detection using anti-5mC and ssDNA samples between 1 nM and 10 μM. The curve from the antibody-based immobilization yields a dissociation constant of 36 nM. It can be concluded that anti-5mC strongly prefers ssDNA samples to dsDNA samples, the opposite of what was observed with MBD-2. This conclusion is reinforced by recent publications (Proll et al., 2006; Rauch and Pfeifer, 2009).

In addition to strand-type (*i.e.*, double stranded vs. single stranded) comparisons, it is important to characterize the OFRR's ability to discriminate small differences in methylation density. Fig. 5(a) compares signals from 10 μM 30-mer DNA samples with either 0 (negative control), 1, 3, or 5 methyl groups per strand. These strands were either fully methylated dsDNA (both strands being methylated), hemi-methylated (only a single strand being methylated), or methylated ssDNA. Several trends are quite clear. Discrimination between strands based on a single methyl group was not observed with MBD-2 because very little single-methylated sample was captured. However, at 3 or 5 methylcytosines fully methylated dsDNA can be clearly distinguished. Some binding also occurred between the methylated ssDNA and hemi-methylated dsDNA samples at 3 or 5 methyl groups per strand, but at a much lower level, as compared to fully methylated dsDNA. Also, there is no statistical difference between the signal from methylated ssDNA and hemi-methylated dsDNA. This is consistent with previous observations in the literature (Hendrich and Bird, 1998). In fact, it is well known that MBD-2 needs to form a dimer with MBD-3 in order to strongly bind hemimethylated DNA (Tatematsu et al., 2000). While these observations have been made before, never have they delivered the ability for quantitative optical label-free analysis. Instead, research has been

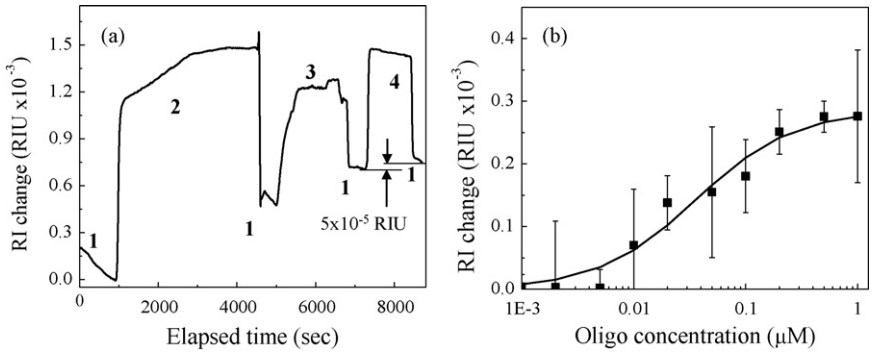


**Fig. 4.** (a) Sensorgram for immobilization of 10 μM dsDNA with 5 methylcytosines with anti-5mC showing very small net shift. Steps: PBS buffer rinse **1**, protein G immobilization **2**, antibody immobilization **3**, and 10 μm ssDNA capture **4**. (b) Titration curves for anti-5mC experiments using 10 μM, 5-methyl ssDNA. The fitted curve yields a $K_d$ of 36 nM for the antibody-based approach.

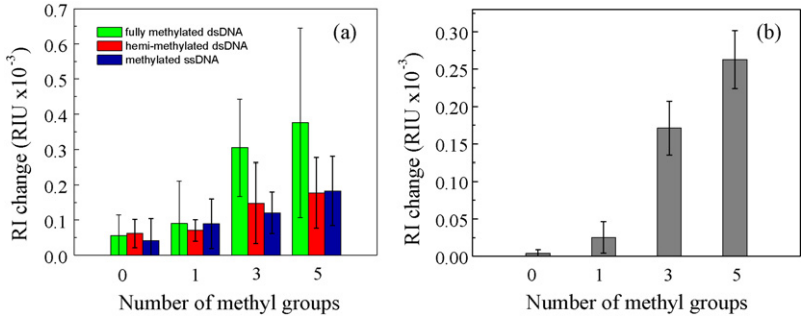


**Fig. 5.** (a) Sensing signal for 10 μM DNA with various methylation states after binding to immobilized MBD-2 proteins. DNA samples were either fully methylated dsDNA (left bars), hemi-methylated dsDNA (middle bars), or methylated ssDNA (right bars). (b) Sensing signal for 10 μM ssDNA with various methylation states after binding to immobilized 5-mC antibodies.

limited to fluorescence-based approaches like microarrays and gel assays.

### 3.3. Anti-5mc assay

In Fig. 5(b), the effect of degree of methylation on the antibody-based approach is examined. We used 10 μM ssDNA with 0, 1, 3 and 5 methyl groups per strand. It is quite clear that in this case, different number of methylation cytosine have significant impact on the ssDNA's affinity for antibody. Using our method, we were able to differentiate between unmethylated and single-methylated ssDNA. Fig. 5(a and b) together demonstrate the obvious difference in the two assays. Methyl binding proteins show much stronger binding affinity for dsDNA and anti-5mC antibodies show much stronger binding affinity for ssDNA. The strengths and weaknesses of these approaches must be considered to determine which is appropriate for a particular application.

## 4. Conclusion

We have developed OFRR label-free sensors for DNA methylation analysis using MBD-2 and anti-5mC as the capture molecule. It is shown that the MBD-2 methods, for most applications, will be preferable due to its ability to bind dsDNA that naturally exists in blood samples. However, the antibody method provides a better capability to identify single methylcytosines. Future efforts will focus on developing this sensor for nucleotide-sequence-specific methylation analysis by expanding our protocol to use both DNA hybridization (for determining sequence specificity) and methyl binding protein recognition (for determining methylation density). Actual DNA samples from blood will also be used to evaluate the clinical utility of the OFRR based methylation analysis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.050.

## References

Baylin, S.B., Herman, J.G., 2000. Trends Genet. 16, 168–174.
Bird, A., 2002. Gene Dev. 16, 6–21.
Bruce, S., Hannula-Jouppi, K., Lindgren, C.M., Lipsanen-Nyman, M., Kere, J., 2008. Clin. Chem. 54, 491–499.
Cox, W.G., Singer, V.L., 2004. Biotechniques 36, 114–122.
Egger, G., Liang, G., Aparicio, A., Jones, P.A., 2004. Nature 429, 457–463.
Fan, X., White, I.H., Zhu, H., Suter, J.D., Oveys, H., 2007. Overview of Novel Integrated Optical Ring Resonator Bio/chemical Sensors. Proc. SPIE 6452, pp. 64520M.
Feng, F., Wang, H., Han, L., Wang, S., 2008. J. Am. Chem. Soc. 130, 11338–11343.
Flusberg, B.A., Webster, D.R., Lee, J.H., et al., 2010. Nat. Methods 7, 461–467.
Fraga, M.F., Esteller, M., 2002. Biotechniques 33, 632–649.
Gitan, R.S., Shi, H., Chen, C.-M., Yan, P.S., Huang, T.H.-M., 2002. Genome Res. 12, 158–164.
Gohring, J., Dale, P.S., Fan, X., 2010. Sens. Actuators B 146, 226–230.
Gorodetsky, M.L., Ilchenko, V.S., 1999. J. Opt. Soc. Am. B 16, 147–154.
Hendrich, B., Bird, A., 1998. Mol. Cell. Biol. 18, 6538–6547.
Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., Baylin, S.B., 1996. Proc. Natl. Acad. Sci. U.S.A. 93, 9821–9826.
Herman, J.G., Umar, A., Polyak, K., et al., 1998. Proc. Natl. Acad. Sci. U.S.A. 95, 6870–6875.
Jacinto, F.V., Ballestar, E., Esteller, M., 2008. Biotechniques 44, 35–43.
Jones, P.A., Baylin, S.B., 2002. Nat. Rev. Genet. 3, 415–428.
Kane, M.F., Loda, M., Gaida, G.M., et al., 1997. Cancer Res. 57, 808–811.
Kelsey, G., Bodle, D., Miller, H.J., et al., 1999. Genomics 62, 129–138.
Li, H., Fan, X., 2010. Appl. Phys. Lett. 97, 011105.
Maki, W.C., Mishra, N.N., Cameron, E.G., et al., 2008. Biosens. Bioelectron. 23, 780–787.
Meehan, R.R., Lewis, J.D., McKay, S., Kleiner, E.L., Bird, A., 1989. Cell 58, 499–507.
Nelson, P.S., Papas, T.S., Schweinfest, C.W., 1993. Nucleic Acids Res. 21, 681–686.
Proll, J., Fodermayr, M., Wechselberger, C., et al., 2006. DNA Res. 13, 37–42.
Rauch, T., Pfeifer, G.P., 2009. In: Tost, J. (Ed.), DNA Methylation: Methods and Protocols, 2nd ed. Humana Press.
Suter, J.D., White, I.M., Zhu, H., et al., 2008. Biosens. Bioelectron. 23, 1003–1009.
Suter, J.D., Howard, D.J., Caldwell, C.W., Shi, H., Fan, X., 2009a. Defense, Security & Sensing. SPIE, Orlando, FL.
Suter, J.D., Howard, D.J., Shi, H., Caldwell, C.W., Fan, X., 2009b. Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE, Minneapolis, MN.
Tatematsu, K., Yamazaki, T., Ishikawa, F., 2000. Genes Cells 5, 677–688.
Valinluck, V., Liu, P., Kang, J.I., Burdzy, A., Sowers, L.C., 2005. Nucleic Acids Res. 33, 3057–3064.
Wade, P.A., 2001. Bioessays 23, 1131–1137.
White, I.M., Oveys, H., Fan, X., 2006. Opt. Lett. 31, 1319–1321.
White, I.M., Zhu, H., Suter, J.D., et al., 2007. IEEE Sens. J. 7, 28–35.
Yu, Y., Blair, S., Gillespie, D., et al., 2010. Anal. Chem. 82, 5012–5019.
Zhu, H., White, I.H., Suter, J.D., Dale, P.S., Fan, X., 2007. Opt. Express 15, 9139–9146.
Zhu, H., White, I.M., Suter, J.D., Zourob, M., Fan, X., 2008. Analyst 133, 356–360.
Zhu, H., Dale, P.S., Caldwell, C.W., Fan, X., 2009. Anal. Chem. 81, 9858–9865.