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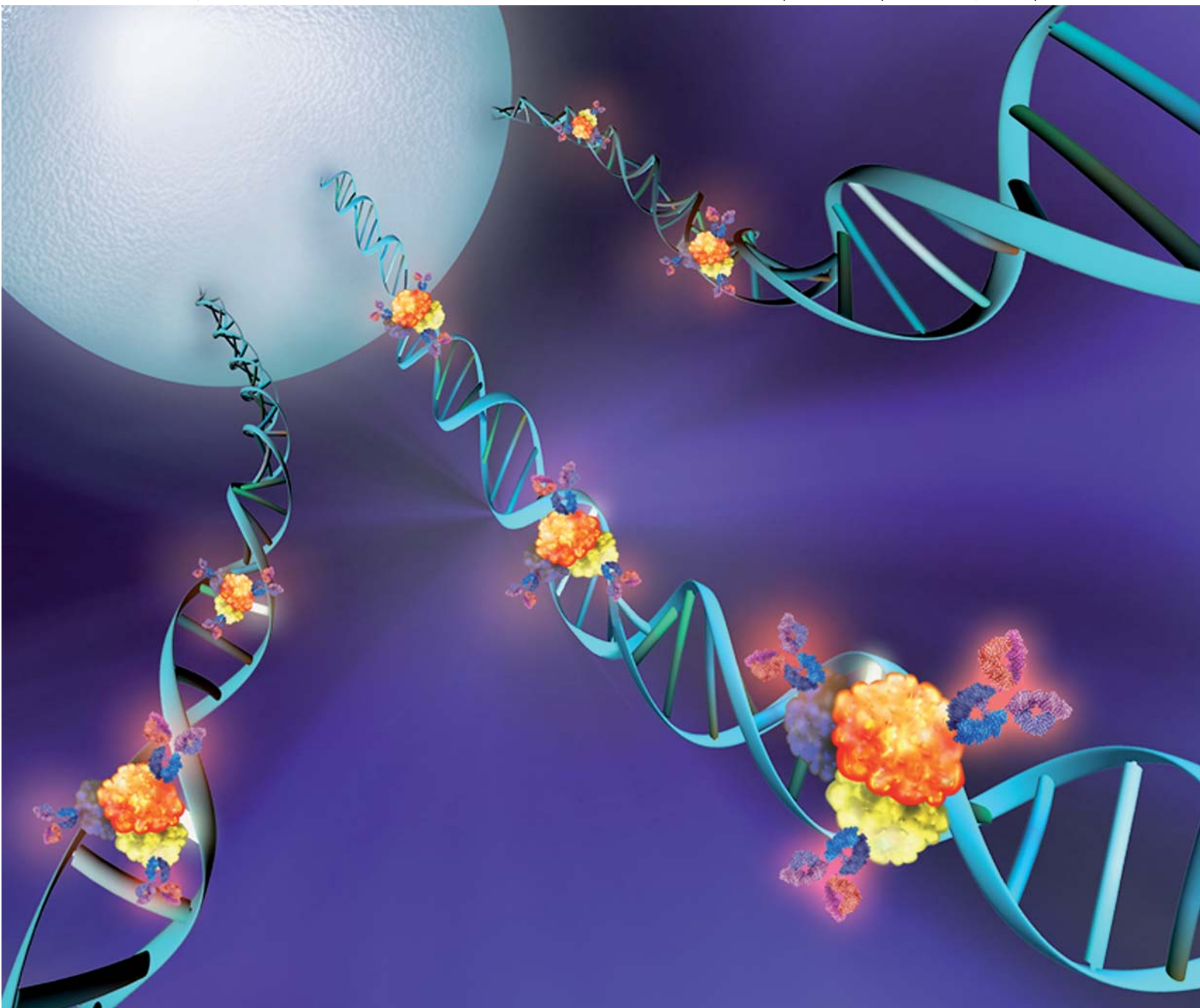
COMMUNICATION
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Bisulfite-free analysis of 5MeC-binding proteins and locus-specific methylation density using a microparticle-based flow cytometry assay†

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DNA methylation analysis is emerging as a new technique with potential capabilities for early cancer detection. However, current state-of-the-art techniques are not easily translatable into routine clinical methods. Herein we describe a bead-based flow cytometry assay which combines DNA hybridization to microparticles with 5MeC-specific proteins/antibodies. These assays can be used to study the binding properties of current and emerging 5MeC-binding proteins and may also have potential in the measurement of 5MeC density in clinical samples for cancer detection.

Epigenetic regulation of gene expression plays a critical role in developing and maintaining eukaryotic cells and organisms.[1,2] Aberrant changes in 5-methylcytosine (5MeC) density in gene promoters ("CpG islands") are correlated with oncogenesis in a range of both solid organ and hematopoietic cancers.[3,4] Both hypermethylation in gene-specific CpG islands and hypomethylation in repetitive sequences (ALU, LINE-1, *etc.*) have been used to distinguish cancers from healthy tissue, sometimes at the early stage.[5,6]

The technology most commonly employed to quantify the location and density of 5MeC is a combination of bisulfite conversion (chemical conversion of unmethylated C → U bases whilst leaving 5MeC unmodified) with sequencing. This method, referred to as "Bisulfite Sequencing" (BS), is capable of distinguishing C/U differences at single-base resolution.[7] Coupled with "Next-Gen" sequencing technologies, the capability for genome-wide methylation mapping will continue to improve.[7] However, bisulfite techniques may be too complicated and costly (8–18 h pre-PCR[8]) for routine clinical use in the near future. Initially, diagnostic tests using methylation analysis are likely to focus on quantifying the relative concentration of 5MeC in a well-characterized DNA sequence[9] (or sequences), for which the extra time and cost of single-base resolution methods are unjustified.

PCR methods have been developed based on the amplification of bisulfite-converted DNA in the presence of methylation-specific primers. Both endpoint assays (methylation-specific PCR or MSP) and real-time (MethyLight) assays have been developed based on this concept, however, they show poor agreement and significant variability when compared to BS.[10] Problems include those associated with bisulfite conversion (time, cost, sample degradation) and the limitation of having to design PCR amplicons around 5MeC-rich primers without dependence on the 5MeC density in-between.

Another class of methylation assays involves selective recognition of 5MeC by anti-5MeC-antibodies (MeDIP—methylated DNA immunoprecipitation) or proteins containing 5MeC-binding domains (MIRA—methylated-CpG island recovery assay). Neither method requires bisulfite, rather a DNA sample is separated into methylated and unmethylated fractions by resin-bound proteins, after which standard PCR analysis can be used to estimate methylation density in specific loci. While NMR studies have been used to investigate the binding mechanisms of some 5MeC-binding proteins (MBPs) to symmetrically methylated dsDNA,[11] there is still much to learn about these interactions. An assay which can rapidly analyse the binding characteristics of MBPs as a function of DNA sequence and structure may be extremely useful in identifying the most appropriate MBP for specific applications.

Herein we report a method for measuring the 5MeC density of a DNA sequence without the need for bisulfite conversion. In its simplest form, we believe it is useful for rapidly screening MBPs to determine DNA sequence/structure-dependent binding affinities. However, there is also the potential for this simple assay to be used in cancer screening strategies to quantify the relative 5MeC density in gene-specific or repetitive loci.
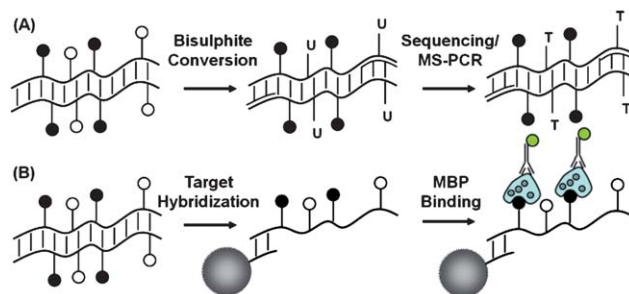

Fig. 1 Bisulfite conversion chemically converts unmethylated cytosine bases (clear circles) to uracils without affecting 5MeC bases (filled circles) (A), however, this is a time-consuming process which results in significant DNA degradation. Methylation-specific PCR involves amplifying bisulfite-converted DNA in the presence of primers which are designed to only amplify sequences containing unconverted 5MeC. However, this places further restrictions on the design of PCR primers and probes, and is only sensitive to 5MeC bases contained within the primer/probe sequences themselves. By combining a microparticle assay with affinity recognition of the 5MeC base (B), we are able to selectively extract DNA loci of interest from a sample, then measure the 5MeC density of the sequence by incubation with MBPs and FITC-labelled secondary antibodies. Relative quantitation of the fluorescent signals is performed rapidly and robustly using flow cytometry.

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An assay using a combination of DNA hybridization and protein recognition was developed to replace bisulfite conversion for locus-specific methylation analysis (Fig. 1). DNA sequences of interest were isolated from the solution by locus-specific hybridization to probe-functionalized microparticles (for detailed characterization of the organosilica microparticles employed in this study, the reader is directed to a range of publications from our group[12–16]). Subsequent incubation with MBPs and FITC-labelled antibodies yielded fluorescent signals indicative of the 5MeC content. Importantly, by incorporating a PEG (polyethylene glycol) linker into the silica microparticles and using a low surfactant concentration in the protein/antibody incubation steps (0.1% Tween-20), we were able to minimise non-specific binding between the MBPs or antibodies and the microparticles. We chose to investigate two different MBPs. GST-MBD2b (Fig. S1†) has previously been reported to bind double-stranded DNA (dsDNA) specifically,[17] while the 5MeC-Ab reportedly binds only to single-stranded sequences (ssDNA).[18] Flow cytometry was used to score the fluorescence intensity of thousands of individual microparticles in each sample to provide a semi-quantitative measure[19] of DNA methylation. We show that the assay can be used to assess and compare binding properties of MBPs using model DNA substrates, identifying previously unknown single-base resolution binding to hemi-methylated DNA. Furthermore, we were able to detect hypomethylation of ALU and LINE-1 sequences in only 10 μL of serum extracted from women with invasive breast cancer in comparison to healthy controls.

Binding of MBPs to symmetrically methylated probe/target combinations yielded results similar to those from a previous study investigating a similar MBP[20] (Fig. S2†) and identical probe/target sequences (Table S1†). However, we hypothesised that MBPs may also bind hemi-methylated DNA, in which only the target sequence contains the 5MeC bases of interest. This would represent a significant advantage for a diagnostic assay as the target 5MeC density could be measured using a completely unmethylated probe sequence. Fig. 2A shows 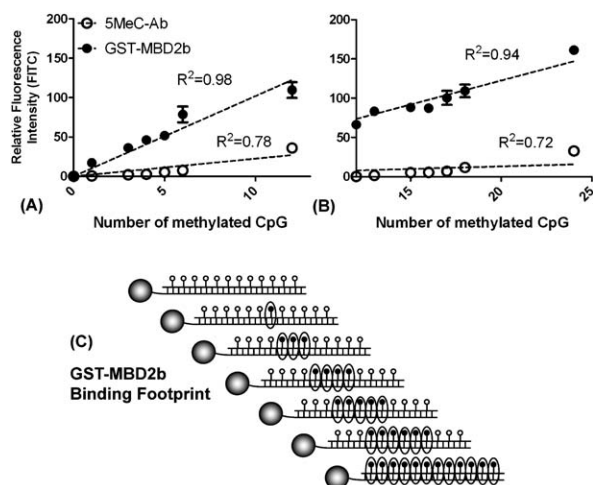that the GST-MBD2b protein could bind a hemi-methylated substrate containing only a single 5MeC, and that increasing the 5MeC density of the target sequence resulted in a linear increase in MBP binding (p-value < 0.0001). Replacing the unmethylated probe (DOM-0-NH2) with the fully methylated sequence (DOM-12-NH2) resulted in a similar linear trend (Fig. 2B) but with a significantly higher y-intercept, suggesting that individual changes in the target methylation density were detectable regardless of probe methylation density. From these results we were able to infer an approximate "footprint" of the protein on the DNA (Fig. 2C), suggesting on average single-base resolution up to at least 12 5MeC bases in the target strand. As expected, the 5MeC-Ab showed low binding to the dsDNA substrates (Fig. 2B) in accordance with previous observations, however, a linear trend was present (p-value = 0.0013 for both) with ~3 methylated bases required for minimum signal detection.

We next investigated MBP binding to microparticle-bound duplexes containing a ssDNA target "overhang" (Fig. 3). We hypothesised that this may result in favourable conditions for 5MeC-Ab binding, based on previous studies reporting a preference for ssDNA targets. Furthermore, assays involving short probes and long targets are more common for DNA hybridization applications, thus it was important to identify which MBP performed best under these conditions. Keeping the total target concentration constant, we mixed the unmethylated and methylated long target sequences (70nt) together in different ratios to make a dilution series. We then incubated these target samples with short (40nt) or long (70nt) probes bound to microparticles and exposed them to the MBPs (Fig. 3). Fig. 3A shows that both MBPs have very similar binding affinities to the samples when a target overhang is present, while the 5MeC-Ab could detect a lower percentage of methylated DNA in comparison to the GST-MBD2b (10% vs. 25%). Fig. 3B shows that the 5MeC-Ab had significantly lower affinity for the substrate containing probe and target of the same length, a result similar to that in Fig. 2.

To investigate the utility of our assay in real clinical samples, we measured ALU and LINE-1 methylation using the 5MeC-Ab, in a small cohort of human serum samples extracted from women with or without invasive breast cancer. We used the 5MeC-Ab instead of the GST-MBD2b because of the lower detection limit (Fig. 3A) and also due to the ALU/LINE-1 assay design involving long ssDA overhang sequences. Hypomethylation of ALU and LINE-1 repetitive elements is a known hallmark of cancer and has been identified in the serum and tissue of many cancers including breast.[6,21–24] The
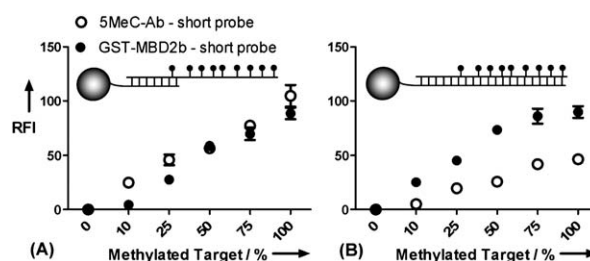

Fig. 2 Hemi-methylated DNA analysis by microparticle assay; (A) linear response curves generated from hemi-methylated DNA using DOM-0-NH2 probe-functionalised microparticles and targets of increasing 5MeC density or (B) linear response curves generated from hemi-methylated DNA using DOM-12-NH2 probe-functionalised microparticles and targets of increasing 5MeC density; (C) schematic of GST-MBD2b binding footprints as indicated by results from (A) and (B).

Fig. 3 ssDNA target overhang analysis by microparticle assay; (A) hybridization of short probes with long targets thus producing a target "overhang"; (B) hybridization of long probes with long targets, producing a duplex of equal length. Note that MBP-specific fluorescence intensity is presented here after normalisation based on the hybridized DNA amount, which varied predictably based on the probe length (Fig. S3†).

histological diagnosis of the samples was confirmed by a pathologist (NBK) and presented in a separate study.[5]

In order to maximize the hybridization efficiency[25] we designed three probes each (~30 bp) for both the human ALU consensus sequence and the human LINE-1 sequence (both >200nt[26,27]), and attached these in a mixture to microparticle batches (Table S1†). As a negative control, we included in the testing panel particles without any conjugated probes, which yielded FITC signals at background levels. Hybridization reactions were performed using only 10 μL serum DNA, the concentration of which were undetectable by nanodrop (i.e. <1 ng μL^{-1}). Therefore we used qPCR to measure the concentration of ALU/LINE-1 in the patient samples (Table S2†) in order to normalize the methylation data for direct comparisons. The normalised methylation data for the clinical samples ("methylation scores") are presented in Fig. 4. Methylation of both LINE-1 and ALU sequences was significantly higher in serum from normal controls than serum from breast cancer patients (p-values < 0.05 using Mann Whitney test), consistent with previous observations in similar studies investigating hypomethylation of repetitive elements in breast cancer.[21–23] Overall, LINE-1 methylation scores were higher than those for ALU regardless of normal or cancer samples, indicative of higher methylation density in LINE-1 sequences which is consistent with other studies investigating hypomethylation in a range of human cancers.

The microparticle assay presented in this study revealed significant MBP binding to hemi-methylated DNA. Furthermore, the binding footprint of the GST-MBD2b protein is apparently much larger in the case of symmetric DNA methylation when compared to the hemi-methylated case. This could be explained by only one of the two potential binding sites[11] being occupied in the latter case, such that less of the protein is interacting with the DNA. Along with evidence of 5MeC-Ab binding to dsDNA, this contradicts previously suggested binding preferences for these MBPs. These assays may therefore prove useful in screening MBPs in a high-throughput manner, changing the composition of the DNA target (ssDNA, dsDNA, symmetric versus hemi-methylation, etc.).

Several elements of the current prototype need further improvement for clinical application. Future work will focus on testing more clinical samples and improving the sensitivity of the method for clinical application. Firstly, whilst hypomethylation of repetitive sequences may be of interest as a general cancer biomarker,

hypermethylation of single-copy sequences in promoters/CpG-islands is tissue and cancer-specific. However, the sensitivity of the assay with respect to the number of target molecules hybridized per microparticle is limiting at this stage. For example, a typical locus is present at 1 copy per 3.3 pg of DNA, therefore 100 ng will contain ~30 000 copies of each locus. The hybridization efficiency of reactions involving PCR products may be as low as 1–10%, leaving only 300–3000 copies hybridized to the microparticles. If 1000 microparticles are used for the reaction and subsequent flow cytometry, there may be as low as 3–30 targets bound to each microparticle. The nominal sensitivity limit for the BDLSR2 flow cytometer is only ~40 molecules of FITC (BD Biosciences). This explains why repetitive sequences are easier to analyse—ALU and LINE-1 sequences are present at ~0.5 to 1×10^6 copies per genome.[6] However, advances in hybridization assay optimization (e.g. minimizing surface density, use of non-native DNA probes to increase K_a, MBPs of increased affinity, etc.) will be investigated in developing the next generation assays. Secondly, incorporation of an internal control measurement for particle-bound DNA would reduce the need for methods such as qPCR or nanodrop analysis. Finally, a key advantage inherent to microparticle technologies is the ease of multiplexing using optically distinguishable beads,[15,16] which will be investigated in future generations of the assay in order to measure the 5MeC density of multiple loci in the same test, which should work to reduce false positives and improve clinical specificity.

We have reported the development of a DNA methylation assay which can quantify the methylation density of a specific DNA locus without the need for bisulfite conversion. Using model systems consisting of a dsDNA duplex containing hemi-methylated CpG-dinucleotides, we identified that both MBPs under investigation could bind to hemi-methylated DNA. We suggest this assay may be of interest in the development of high-throughput cancer detection assays based on ALU/LINE-1 methylation and also in the development of research techniques focusing on quantitative analysis of the binding characteristics of MBPs.
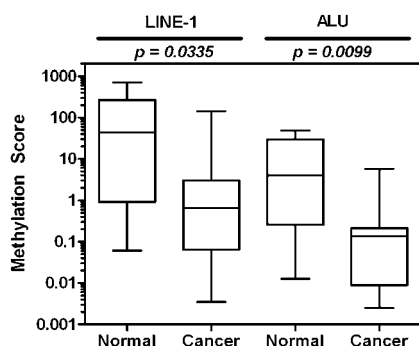
Acknowledgements

Fig. 4 ALU and LINE-1 microparticle-based methylation scores for serum extracted from women with and without invasive breast cancer using 5MeC-antibody as the detection agent. Note that box indicates interquartile range (25th to 75th percentile) and whiskers indicate max/min values.

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