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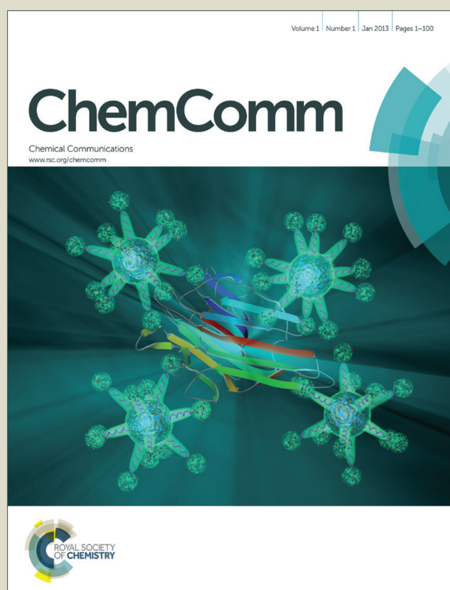

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COMMUNICATION

Measuring whole genome methylation via oxygen channelling chemistry

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Successful use of demethylating drugs in cancers underscores the need to analyse whole genome DNA methylation in the clinic. Unfortunately, current methods are difficult to perform and require large amounts of DNA input. Herein we describe the first application of oxygen channelling chemistry for detecting DNA methylation which requires 2 hours to perform, 10-fold less input material than conventional methods, is sensitive to 5% difference in methylation and able differentiate samples before and after demethylating treatment.

Epigenetic changes in DNA are gaining interest as disease biomarkers.[1-3] The most studied form of epigenetic DNA change is the methylation of cytosine in cytosine/guanine (CpG) dinucleotides. Approximately 70-80% of all CpGs in the genome are methylated.[4] However, in regions of high CpG densities or CpG islands (e.g. gene promoter regions), aberrant methylation can lead to diseases such as cancer. Azanucleosides drugs such as 5-aza-2'-deoxycytidine (5-Aza) have been used successfully as a therapy for reactivating silenced genes in epigenetic diseases.[5-7] However, characterizing the mechanism of action and tracking the effectiveness of demethylating drugs, i.e. patient response, is difficult with current technologies.

Traditional approaches of evaluating demethylation include High Performance Liquid Chromatography (HPLC),[8] mass spectrometry (MS)[9] and bisulfite conversion[10, 11] of DNA followed by some form of sequencing.[12-16] More recently, affinity capture approaches using Methyl-Binding Domain (MDB) proteins or antibodies raised against 5-methylcytosine have been used to enrich for methylated DNA prior to Next Generation Sequencing (NGS).[17] While excellent for research, such approaches are not feasible for routine diagnostics. Therefore, new methods are needed to facilitate regular monitoring of patient response to demethylating therapies.

The oxygen channelling chemistry[18] proximity assay has been successfully employed for detecting protein/protein and protein/DNA interactions and has various additional benefits over standard ELISA such as faster, easier protocols with better sensitivity and lower input sample requirements.[19] This approach however, has not been adapted for detecting DNA methylation. As mentioned earlier, MBDs are particularly useful in specifically differentiating methylated from unmethylated DNA and have been used successfully in many whole methylome studies.[17] In addition, unlike antibodies raised against methylated cytosines on single-stranded DNA, MBDs, particularly MBD2a, are highly selective for methylated cytosines in native double-stranded DNA[20], hence are relatively more convenient to use. Therefore, it is conceivable that coupling the selectivity and convenience of MBDs with the benefits of an oxygen channelling platform could result in a sensitive and rapid detection strategy for whole genome methylation that may be useful in routine diagnostic applications such as monitoring patient response to demethylating drugs.

Our assay relies on the ability to bring two particle sets (acceptor and donor) into close enough proximity to enable an oxygen channelling chemistry[21] via a MBD2a/DNA-biotin/streptavidin bridge (Scheme 1). To realize the methodology for detecting DNA methylation, DNA is first enzymatically fragmented and tagged with biotin (refer to ESI for detailed methods). Using the selected endonucleases, the resulting DNA fragments are approximately 128 bases (~44 nm) long on average. This length is within the effective distance for efficient oxygen channelling[18] and hence could potentially detect all methylation sites on the DNA fragment.

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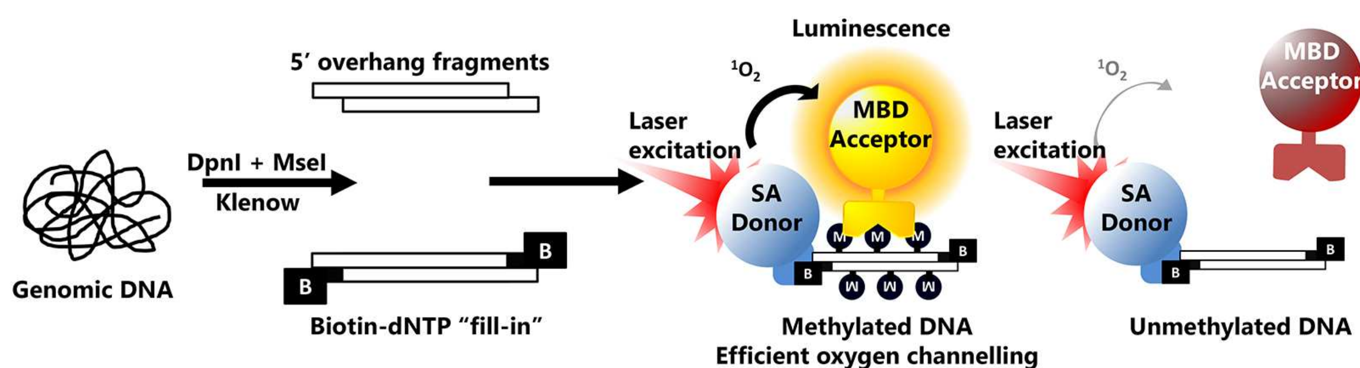

Scheme 1. Conceptual scheme of the proximity assay. Genomic DNA is first enzymatically fragmented and labelled with biotin. Biotinylated DNA fragments then serve as substrates for both MDB acceptor and streptavidin (SA) donor particles. Only in the presence of methylated DNA can SA donor particles get in close proximity of MDB acceptor beads to enable efficient oxygen channelling chemistry to generate a luminescence signal.

The enzyme-modified DNA is then reacted with both MBD-conjugated (acceptor) and streptavidin-conjugated (donor) particles. Only in the case of methylated DNA can both populations of particles come into close enough proximity to enable efficient oxygen channelling chemistry. The resulting luminescent is then in turn, proportional to the amount of methylated DNA in the sample.

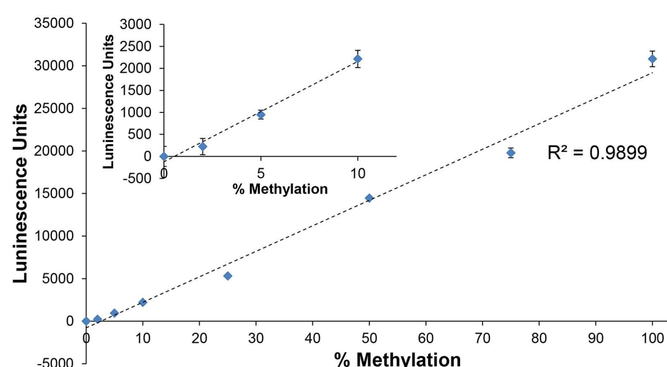

Figure 1. Calibration plot showing the positive correlation ($R^2 = 0.989$) between luminescence response with increasing % methylation. Insert is a magnification of the response curve between 0% to 10% methylation. Errors bars represent standard deviation over 3 independent experiments.

To demonstrate the feasibility of the assay, we first used, as a proof-of-concept, a 300 bp DNA strand titrated at various proportions of methylated to unmethylated strands (i.e. % methylation). Figure 1 clearly shows the positive correlation ($R^2 = 0.989$) between % methylation and oxygen channelling chemistry mediated luminescence. For a 400 pg sample, at 1:2 ratio of acceptor to donor particles, we could detect as low as 5% methylation suggesting a sensitivity to 5% differences in methylation levels. This assay also had a RSD of 4.4% (n = 3) indicating very high reproducibility. The oxygen channelling chemistry is also a very rapid readout method that is completed within 10 mins of mixing the reactants (ESI Fig. S1 and S2). While not yet tested in this study, adjusting the acceptor to donor bead ratio and exploiting the "hooking effect[22]", one may be able to skew the assay towards the either the methylated or unmethylated DNA species and thus potentially tuning the assay sensitivity.

Traditional HPLC and MS methods also detect total methylation. However, it is difficult to directly compare them with our assay because both HPLC and MS approaches consider all cytosines in the genome whereas our assay is a MBD enrichment approach that only considers a subset of cytosines and therefore give different measurements of methylation. However, a recent study using MBDs and antibodies against methylated cytosines but coupled to a flow cytometry readout, could only detect 10% and 25% methylated samples respectively.[23]

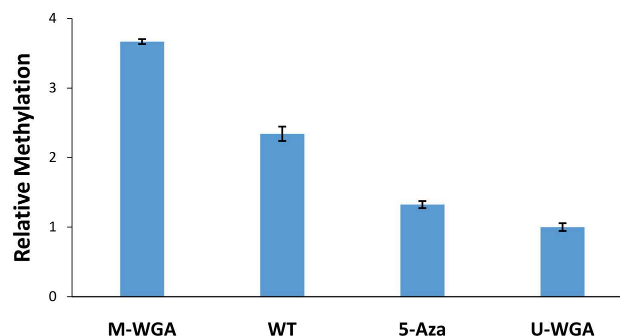

Figure 2. Distinguishing samples before (WT) and after demethylating treatment (5-Aza). M-WGA: methylated control. U-WGA: unmethylated control. Figure Caption. Error bars represent standard deviation over 3 independent experiments

Next, to demonstrate applications in clinical scenarios, we performed the assay on more complex DNA systems. Whole genome amplified (WGA) DNA before and after *in vitro* methylation was first trialled to assess assay performance on more complex DNA samples (Fig 2, S2). To this end, we could clearly distinguish between methylated and unmethylated WGA DNA from 50 ng of starting material. Finally, we wanted to see if the assay could also distinguish genomic DNA derived from human cancer cells lines before and after 5-Aza. Figures 2 and S2 clearly show the difference in signal before and after treatment with the demethylating drug thus demonstrating its potential for tracking patient response to such drug treatments. By generating a calibration plot with the methylated (M-WGA) and unmethylated (U-WGA) controls, 5-Aza treated cells was estimated to be 11.5% methylated in contrast to untreated cells (WT) that were

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approximately 48.8% methylated, consistent with the demethylating effects of 5-Aza treatment. In addition, our estimate for total methylation in naïve cells was also consistent with the literature[24] thus indicating the accuracy of our assay.

The traditional HPLC[8] and MS[9] approaches while useful, have difficult sample preparation and require high amounts of input DNA (micrograms) thus severely limiting its adoption in routine diagnostics. In contrast, our approach uses at least one order of magnitude lesser DNA (~50 ng) of starting material and is completed in approximately 2 hours.

Conclusions

In conclusion, we have demonstrated for the first time, an oxygen channelling proximity assay for simple and rapid detection of total genomic DNA methylation. The high sensitivity, ease and very low sample requirements of our approach is highly suited for routine diagnostics unlike traditional methods. The assay also has immediate clinical applications in tracking patients' response to demethylating agents.

Notes and references

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