Optical biosensing strategies for DNA methylation analysis

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\textbf{ABSTRACT}

DNA methylation is an epigenetic modification of DNA, where a methyl group is added at the fifth carbon of the cytosine base to form 5 methyl cytosine (5mC) without altering the DNA sequences. It plays important roles in regulating many cellular processes by modulating key genes expression. Alteration in DNA methylation patterns becomes particularly important in the etiology of different diseases including cancers. Abnormal methylation pattern could contribute to the pathogenesis of cancer either by silencing key tumor suppressor genes or by activating oncogenes. Thus, DNA methylation biosensing can help in the better understanding of cancer prognosis and diagnosis and aid the development of therapies. Over the last few decades, a plethora of optical detection techniques have been developed for analyzing DNA methylation using fluorescence, Raman spectroscopy, surface plasmon resonance (SPR), electrochemiluminescence and colorimetric readouts. This paper aims to comprehensively review the optical strategies for DNA methylation detection. We also present an overview of the remaining challenges of optical strategies that still need to be focused along with the lesson learnt while working with these techniques.

1. Introduction

DNA methylation is one of the important epigenetic alterations, which results from the enzymatic addition of a methyl group at the fifth carbon of the cytosine ring (5mC) (Bird, 2002; Tucker, 2001). Although DNA methylation does not change the sequence of the DNA strands, the level and distribution patterns of 5mC across the genome regulate the genomic imprinting (Li et al., 1993; Constancia et al., 1998) and X-chromosome inactivation (Mohandas et al., 1981; Robertson and Jones, 2000). DNA methylation is also essential in maintaining genomic stability to prevent the onset of somatic mutations (Jones and Gonzalgo, 1997; Robertson and Jones, 2000; Taleat et al., 2015). The methyl group of 5mC can be removed via demethylation pathways, which enables cells to manage their own 5mC levels as well as to control gene expression and protein turnover depending on their needs. In this way, information encoded by the methylation pattern across the genome defines the cell’s identity and function, which becomes crucial in the study of embryonic development, cell differentiation, and onset of many diseases including cancer (Gonzalgo and Jones, 1997; Robertson and Jones, 2000; Kulis and Esteller, 2010).

Detection of 5mC in eukaryotic DNA was first described by Hotchkiss in 1948 and later by Wyatt in 1951 (Hotchkiss, 1948; Wyatt, 1951). In cancers, a gradual loss in cytosine methylation levels throughout the genome (i.e., global hypomethylation) affects 5mC level that are evenly distributed (average 1 CpG in every 150 bp) (Ehrlich, 2002; Kulis and Esteller, 2010). Despite this loss, a hallmark of the cancer development is the simultaneous acquisition of increasing 5mC levels in the genome known as hypermethylation (Aggerholm et al., 1999; Ehrlich, 2002; Kulis and Esteller, 2010). Hypermethylation occurs in cytosines that are clustered in CpG rich regions located at the promoter and the first exon regions of various tumor suppressor genes and proto-oncogenes and can in turn causes their transcriptional silencing (i.e., regional hypermethylation). However, hypomethylation activates transcription of proto-oncogenes, proteins involves in genomic instability and metastasis in cancer (Herman and Baylin, 2003). Generally, the presence of 5mC in a genomic DNA undergoes multiple epigenetic changes such as chromatin condensation, stabilization of chromosomes, genomic imprinting and gene silencing (Reik and Dean, 2001). Also, by altering methylation regionally at non-clustered sites, cancer cells can reprogram their signalling pathways to ensure their
own survival and acquire the ability to invade other tissues. Thus, the DNA methylation landscapes for cancer and healthy genomes are very different, not only in their global levels but also in their distribution across the genome (Ehrlich, 2002; Kulis and Esteller, 2010).

There is an increasing evidence that the methylation status of certain genes in different cancers provide independent information on patient prognosis (Tang et al., 2000). For instance, Zou et al. (2002) provided strong evidences that the detection of p16 methylation in the serum samples of patients with colorectal carcinoma was significantly associated with advanced pathological stages (stage 3 or 4). Also, in colorectal carcinoma, methylation is a common cause of microsatellite instability (MSI) as detected by loss of immunohistochemistry. Sporadic MSI+ colorectal carcinomas result from methylation of mismatch repair genes (Pakhneshan et al., 2013). Similarly, methylation of SHOX2 in lung cancers (Dietrich et al., 2012) and PITX2 promoter methylation in prostate (Dietrich et al., 2013) and breast carcinomas (Nimmrich et al., 2008) were correlated with patient prognosis. Therefore, accurate quantification of methylation level at whole genome and regional levels not only provides a characteristics signature of gene function but also gives a better understanding about the clinical and prognostic roles of DNA methylation in different cancers.

Over the past several decades, various approaches have been extensively developed to quantify the level of the DNA methylation in research and clinical applications (Fig. 1.) (Singer-Sam et al., 1990; Frommer et al., 1992; Herman et al., 1996; Gonzalez and Jones, 1997; Stach et al., 2003; Gao et al., 2005; Weber et al., 2005; Kristensen et al., 2008; BonDurant et al., 2011; Hibi et al., 2011). For example, in 1992 Frommer et al. (1992) introduced the bisulfite conversion method for the analysis of 5mC. Since then, this method has been widely used in clinical applications. Several other methods such as methylation specific (MS) polymerase chain reaction (MS-PCR) (Herman et al., 1996), real-time PCR (BonDurant et al., 2011), quantitative MS-PCR (Hibi et al., 2011), methylation-sensitive single nucleotide-primer extension (MS-SnUPE) (Gonzalgo and Jones, 1997), methylhydrol (Eads et al., 2000), sensitive melting analysis after real-time MS-PCR (Kristensen et al., 2008) and microarray based (Yan et al., 2001; Gao et al., 2005) methods have also been adopted with the bisulfite conversion method to achieve better quantification of 5mC. Most of these methodologies typically require large sample volumes due to DNA degradation during bisulfite conversion and are often limited by low amplification efficiency and PCR bias. Microarray based methods overcome the need for sequencing but still rely on bisulfite conversion and PCR amplification. To avoid the use of bisulfite conversion and PCR amplification, enzyme-linked immunosorbent assay (ELISA) based methylation assays (So et al., 2014; Kurdyukov and Bullock, 2016) were proposed. These assays were relatively simpler but affected by low sensitivity and require multiple external controls for quantitative analysis. To address these issues, methyl binding domain (MBD)
based assays were developed, where MBD protein or other specific antibody (i.e., MeDIP-seq) were used to selectively detect the methylated CpG sites (Weber et al., 2005) within the target DNA sequence.

Besides bisulfite conversion and MBD based methods, high performance liquid chromatography (HPLC) and mass spectroscopy (MS) have also been used for the direct quantification of DNA methylation (Ramasahoye, 2002; Liu et al., 2009; Armstrong et al., 2011; Lisanti et al., 2013). Although HPLC and MS based methods detect DNA methylation directly, they also require a large amount of input DNA which limit their use in routine clinical applications. Other MS based strategies including matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Hillenkamp et al., 1991) have also been reported for the simultaneous quantification of DNA methylation (Friso et al., 2002; Tost et al., 2003; Le et al., 2011; Hu et al., 2013; Le et al., 2011; Humeny et al., 2003).

In recent years, a number of fluorescence readout based methods (Duan et al., 2010; Taleat et al., 2015; Ma et al., 2015; Wang et al., 2016), have successfully been used to quantify DNA methylation. Among these fluorescence readouts, fluorescence resonance energy transfer (FRET) is most commonly used optical readout method in DNA methylation analysis due to its superior sensitivity (Bailey et al., 2010; Cao and Zhang, 2012; Chen and Wang, 2013; Dadmehr et al., 2014; Duan et al., 2010). Semiconductor quantum dots (QDs) have also been adapted in fluorescence based methylation assays (e.g., methylation sensitive (Ms)-FRET) to further improve the assay performance considering the fact that QDs offer broader absorption spectra and show higher detection efficiency when compared to those of conventional fluorophores (Clapp et al., 2006; Resch-Genger et al., 2008; Bailey et al., 2009; Ma et al., 2015). Other optical readout methods such as Raman spectroscopy (Wang et al., 2016), surface plasmon resonance (SPR) (Sina et al., 2014), electrochemiluminescence (ECL) (Zhao et al., 2015) and colorimetry (Wang, et al., 2015a) have also been widely used for analyzing DNA methylation (Kurita et al., 2012; Li et al., 2012; Zhao et al., 2015).

The advancements of DNA methylation detection strategies have been reviewed recently (Zhang et al., 2015; Taleat et al., 2015; Hernandez, 2013). For instance, Zhang et al. (2015) reviewed the use of conventional approaches in methylation analysis whereas Hernandez et al. (2013) discussed PCR based strategies. Later, Taleat et al. (2015) reported a more comprehensive review on the current methylation detection techniques evolved till 2014. To the best of our knowledge, till date, no review has solely covered the available optical techniques for DNA methylation detection. The present review gives a brief outline of the bisulfite sequencing based methylation studies followed by a comprehensive overview of the recent advancements in optical detection strategies for methylation analysis. More importantly, we highlights the technical challenges that have been addressed to make each of the techniques suitable for DNA methylation analysis. In the end, we also put our perspectives on the future direction of the field.

2. DNA methylation analysis strategies

2.1. Sodium bisulfite treatment based techniques

Until 1992, the regular DNA sequencing technologies were unable to distinguish cytosine and 5mC. To solve this problem, complex sequencing strategies based on different digestion and chemical cleavage steps were proposed (Church and Gilbert, 1984; Saluz and Jost, 1989). Most of these strategies were limited by the low sensitivity. Additionally, the sequencing results were obtained by averaging a population of DNA. Therefore, these methods failed to detect methylation level in a small volume of sample. The bisulfite sequencing method, introduced by Frommer and colleagues in 1992, can quantitatively distinguish 5mC from cytosine (Frommer et al., 1992). The method was based on the sodium bisulfite-mediated conversion of cytosine to uracil in single-stranded DNA, where cytosine is converted to uracil but 5mC remains unchanged. After PCR amplification and sequencing, uracil derived from the cytosine is read as thymidine. On the other hand, as 5mC is immune to bisulfite conversion, it is counted as cytosine. This method has been successfully employed to develop several bisulfite sequencing based methylation assays such as MS-PCR and Ms-SnUPE have been developed (Herman et al., 1996; Gonzalez and Jones, 1997). In MS-PCR, bisulfite treated DNA was PCR amplified using two methylation specific primers. As amplification was done by 5mC specific primers, the presence of amplified 5mC in the PCR product confirmed the methylation of target sequence. MSPCR successfully eliminated the common PCR bias, however, it could not analyze the methylation quantitatively (Herman et al., 1996). A follow up strategy known as Ms-SnUPE was able to quantify DNA methylation by electrophoretic separation and radiation analysis of sample (Gonzalez and Jones, 1997).

Bisulfite treated DNA was further studied for methylation analysis by (i) pyrosequencing technology (Tost and Gut, 2007), (ii) melting temperature based sequence-free methods (e.g., methylation-specific denaturing gradient gel electrophoresis (MS-DGGE, Aggerholm et al., 1999), methylation-specific melting-curve analysis (MSMCA, Worm et al., 2001) and high-resolution melting based assay (Wojdacz and Dobrovic, 2007)) and (iii) treating with restriction enzymes (e.g., combined bisulfite-restriction analysis (COBRA), Xiong and Laird, 1997). Pyrosequencing technology was later adapted in next generation sequencing (NGS) technology (e.g., reduced representation bisulfite sequencing (RRBS)) for high throughput and cost-effective analysis (Meissner et al., 2005; Taylor et al., 2007; Hirst and Marra, 2010; Gu et al., 2010; Li et al., 2010; Gu et al., 2011).

2.2. Methyl CpG binding domain (MBD) based techniques

Bisulfite sequencing of DNA derived from heterogeneous sample can result in an altered representation of original methylation pattern (Warzecke et al., 1997). This limitation was overcome by the successful use of MBD proteins in methylation assays (Hiraoka et al., 2012; Shiraishi et al., 2004). The MBD based methods are also less vulnerable to DNA damage (one of the disadvantages of bisulfite sequencing method) (Seguin-Orlando et al., 2015). Generally, MBD proteins (i.e., MeCP2, MBD1, MBD2, MBD3 and MBD4 in mammals) consist of a special domain (i.e., MBD) which has very strong affinity for 5mC region of DNA. Thus, MBD proteins recognize and bind methylated CpG region in double-stranded DNA (dsDNA) with high affinity (nanomolar Kd) (Taleat et al., 2015; Kurdyukov and Bullock, 2016). In 2004, Shiraishi and colleagues used MBD protein to detect 5mC by column chromatography, and demonstrated that the binding efficiency between MBD protein and 5mC is directly proportional to the level of the 5mC present in the genome (Shiraishi et al., 2004). However, this method was limited by the large nonspecific binding to MBD proteins. This nonspecific binding can be avoided using restriction enzymes in MBD based assays. For example, in a restriction enzyme based assay referred to as COMPARE-MS (combining methylated-DNA precipitation with methylation-sensitive restriction enzyme), target DNA was first digested by methylation-sensitive restriction enzymes and then precipitated by MBD polypeptides immobilized on a magnetic bead followed by RT-PCR for quantitative detection (Yegnasubramanian et al., 2006).

MBD proteins or MBD specific antibodies have also been used to enrich the CpG islands for large scale analysis of global methylation. For example, for detecting DNA methylation in genomic DNA, CpG regions were enriched by MBD specific antibodies (e.g., MeDIP-seq) (Weber et al., 2005) or methyl binding domain protein (e.g., MBD-seq) (Aberg et al., 2012), which were then detected using microarray or NGS based readouts. The accuracy of these approaches was, however, affected by several downstream amplifications steps. This drawback was avoided in an improved protocol, where a DNA-mediated floccula-
method. In 1979, Cedar and colleagues reported the cleavage of DNA digested selectively using the methylation assay, the region of interest of DNA (i.e., 5mC) can be evaluated of DNA methylation (Wee et al., 2015b).

**Table 1** Optical detection strategies for DNA methylation.

<table>
<thead>
<tr>
<th>Detection strategy</th>
<th>Starting materials</th>
<th>Note on methylation biosensing</th>
<th>Detection limit</th>
<th>Advantages and limitations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>Synthetic DNA and human lung cancer cell line</td>
<td>Circular padlock probes formation in presence of 5mC, hyperbranched rolling circle amplification.</td>
<td>0.8 pM</td>
<td>Highly sensitive, alternative PCR free amplification, restriction enzyme free. Complex operation, multi-step process.</td>
<td>(Cao and Zhang, 2012)</td>
</tr>
<tr>
<td>Colorimetry</td>
<td>WGA DNA and HeLa and DuCap cell line</td>
<td>MBD based magnetic enrichment, streptavidin coated horse radish peroxidase as color reporter.</td>
<td>37.5 picogram</td>
<td>Naked eye assessment, both global and site specific methylation detection, low sample volume requirement. Require multiple controls, require methyl binding domain (MBD).</td>
<td>(Wue et al., 2015a)</td>
</tr>
<tr>
<td>Quantum dots (QDs) in FRET</td>
<td>Synthetic DNA and lung cancer tissue</td>
<td>Water soluble QDs as FRET donor methylation sensitive restriction, alexa Fluor −647 (FRET acceptor) giving FRET signal in presence of 5mC.</td>
<td>Not mentioned</td>
<td>Multiplexing capacity, simultaneous assay monitoring, photoresistant sensor. PCR bias, restriction digestion required, expertise required.</td>
<td>(Ma et al., 2015)</td>
</tr>
<tr>
<td>Surface Enhanced Raman Spectroscopy</td>
<td>Synthetic DNA and lung cancer tissue</td>
<td>Gold nanoparticle modified capture probe as SERS substrate, cyanine dye as SERS label, single base extension reaction (in presence of 5mC).</td>
<td>3 pM</td>
<td>Highly sensitive and accurate, multiplexing ability, real time analysis. Expertise required, toxicity of SERS label, larger fluorophores.</td>
<td>(Hu and Zhang, 2012)</td>
</tr>
<tr>
<td>Surface Plasmon Resonance (SPR)</td>
<td>Synthetic DNA and lung cancer tissue</td>
<td>Sandwich recognition by antibody and MBD protein, increased refractive index in presence of 5mC.</td>
<td>5 pM</td>
<td>Real time and label free detection, explain sample kinetics and thermodynamics during binding. Large sample volume required, nonspecific bindings.</td>
<td>(Pan et al., 2010)</td>
</tr>
<tr>
<td>Electrochemiluminescence based</td>
<td>Synthetic DNA and DNA bacteriophage</td>
<td>Acetylcholine esterase labeled anti 5mC antibody, ECL emission from Ru (bpy)32+ luminophore.</td>
<td>0.18 pmol</td>
<td>Highly sensitive, PCR-free, digestion-free, bisulfite conversion-free. Antibody requirement.</td>
<td>(Kurita et al., 2012)</td>
</tr>
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tion assay was coupled with the MBD enrichment step for naked eye evaluation of DNA methylation (Wee et al., 2015b).

### 2.3. Enzymatic digestion based assays

Enzymatic digestion based methylation assays have been developed long before the inception of bisulfite conversion based assays (Bird, 1978; Cedar et al., 1979). Generally in an enzymatic digestion based methylation assay, the region of interest of DNA (i.e., 5mC) can be digested selectively using specific restriction enzymes (e.g., endonuclease MspI), followed by the quantification via a suitable readout method. In 1979, Cedar and colleagues reported the first approach to detect DNA methylation directly in eukaryotes by using radiolabeled MspI and HpaII restriction enzymes (Cedar et al., 1979). In this method, target DNA was first digested with MspI and HpaII enzymes, where MspI cleaved the target region of DNA (irrespective of whether the sequence is methylated or not) and HpaII cleaved only the unmethylated target region. These digests were then run on gel electrophoresis for the visual separation of methylated and unmethylated DNA.

Due to the ability of sensitive selection of methylated regions, enzymatic digestion based assays have been adapted in many widely used regional methylation detection platform including RGLS (Costello et al., 2002; Rush and Pfaff, 2002). Though enzymatic digestion based assays are very promising, their applications in point-of-care diagnostics are limited by radiation hazards and requirements of expertise.

### 2.4. Major drawbacks of bisulfite based techniques and current requirements

Despite being the most widely used DNA methylation analysis method, bisulfite based methods have some common drawbacks. One of the drawbacks is the DNA fragmentation which could result chimeric product, making amplification of long fragments difficult. Another drawback is their uncertainty of complete conversion. This could be the result of either failure of deamination of cytosine or an inappropriate conversion of 5mC to thymine (Genernejus et al., 2008). The failure of deamination of cytosine in bisulfite conversion thus gives an altered assessment of DNA methylation. On the other hand, due to the inappropriate conversion of 5mC to thymine, 5mC is mistaken as unmethylated which also gives biased representation of the methylation status. These limitations of bisulfite conversion along with the use of radioactive materials, limited read length of DNA, the need for cumbersome procedures and specialized instruments limit their wide applications in screening of DNA methylation in clinics. Although sequencing based method such as NGS is highly cost-effective and can potentially analyze methylation at the nucleotide level in the whole genome, it has significant bioinformatic challenges (i.e., due to the large sequencing data management) (Hirst and Marra, 2010). Various melting temperature based sequence-free methods including MS-DGGE and MSMCA are unable to precisely detect methylation pattern at single base level. This drawback was resolved by the development of the high-resolution melting (HRM) method. However, HRM method is not suitable for methylation screening of heterogeneous samples. Generally, clinical samples contain a heterogeneous population of cells. This diverse variability in cellular composition can heavily interfere the methylation analysis, leading to high differences between presumably two identical tissue samples. Therefore, a method that uses small volumes of sample (low number of homogenous cells) is highly desirable for overcoming complication in extracting accurate methylation information. On the contrary, analysis of large cohort samples is also required to understand prognosis and diagnosis of many diseases including cancer. Therefore, robust and cost-effective methods are needed for analyzing a large cohort of samples. Currently a significant
level of advances has been made along this line and as a result, a number of optical based DNA methylation readout strategies have been developed which offer several advantages over conventional methods including low sample requirement, high-throughput screening, sensitive limit of detection (LOD), real-time analysis, label-free detection, PCR bias free approach, heterogeneous methylation screening, naked eye evaluation etc. (Table 1).

3. Optical detection of DNA methylation

Fluorescence, electrochemiluminescence, SERS, SPR, and colorimetric readouts are the most commonly used optical detection methods in methylation assays (Fig. 2, Table 1). In this section, these developments will be discussed.

3.1. Fluorescence based techniques

Among many other fluorescence detection methods, FRET is the most widely used method for detecting DNA methylation as FRET based readouts offer high sensitivity in methylation detection due to their less susceptibility to the nonspecific interferences. In FRET, a donor fluorophore from an electronically excited state gives electron to an acceptor chromophore resulting change of fluorescence intensity. This energy transfer happens due to the dipole–dipole interactions between a donor and an acceptor, where the FRET probability is inversely proportional to the distance between donor and acceptor by the sixth power (Miyawaki, 2003; Freeman and Willner, 2012). In DNA methylation analysis, several donor and acceptor fluorophores including cationic conjugated polymers (CCPs) (Feng et al., 2008; Yang et al., 2012), quantum dots (QDs) (Ma et al., 2015) and upconversion nanoparticles (UCNPs) (Wu et al., 2016) have been used. Usually, in FRET based methylation assays, donor fluorophores specifically can bind the target (i.e., 5mC). When donors are excited at their absorption spectra, they transfer energy to the acceptor molecules. By obtaining transferred energy, acceptor molecules then results a significant fluorescence amplification. Since unmethylated DNA do not bind to 5mC specific donor molecules, they do not transfer energy to the acceptor molecules resulting no FRET signal (Fig. 2).

Over the past several years a number of FRET based methylation analysis methods have been developed. In most of these methods, CCPs are used as FRET donors as they act as powerful transducer which triggers electron transfer from acceptor fluorescein through FRET. Since fluorescence intensity is obtained from both CCP and fluorescein, it gives a strong optical amplification allowing sensitive detection of DNA methylation (Feng et al., 2008; Duan et al., 2010; Yang et al., 2012). For instance, it was successfully employed to detect as low as 1% methylation status of CpG sites in human colon cancer cells (Feng et al., 2008). Duan et al. (2010) demonstrated another CCP based FRET assay for the detection of DNA methylation (Fig. 3A.). In this method, bisulfite converted DNA was initially amplified by PCR. Single base extension of the amplified DNA samples were then performed using Taq polymerase in the presence of fluorescein labeled deoxyguanosine triphosphate (dGTP-Fl) and methylation-specific probe. The dGTP-Fi specifically recognized and bound complementary probe of methylated DNA but did not attach with the unmethylated probe. Consequently, after the addition of cationic polymer PFP1a, only methylated DNA sample resulted a strong FRET readout leaving unmethylated counterpart irresponsive. This assay was validated in the genomic DNA sample extracted from human colon cancer cell line, HT29.

In recent years, quantum dots (QDs) based FRET donors have also been used to improve the sensitivity of the fluorescence based
methylation assays (Bailey et al., 2010; Ma et al., 2016). QD is a semiconductor nanocrystal which stays in a core shell structure. The fluorescence emission from QDs is dependent on the size of the crystals and is unaffected by photobleaching. Additionally, while many other organic fluorophores are affected by the narrow absorption spectra, QDs provide wide absorption spectra along with narrow emission.

Fig. 3. (A) Schematic representation of FRET based assay. Bisulfite converted and PCR amplified-methylated DNA was extended with Taq polymerase in the presence of dGTP-Fl. In the detection step, methylation-specific probe and PFP1a resulted strong FRET signal whereas unmethylated sample produced no FRET response. (B) An ECL based DNA methylation assay. The acetylcholinesterase labeled anti methylcytosine antibody was used to quantify DNA methylation by measuring ECL emission from Ru(bpy)32+ luminophore. Reproduced from Duan et al. (2010) and Kurita et al. (2012) with permission from The American Chemical Society.
spectra and high extinction coefficient (Clapp et al., 2006; Resch-Genger et al., 2008; Ma et al., 2015; Ma et al., 2016). In 2009, Bailey and colleagues developed a QD based FRET analysis for DNA methylation referred to as MS-qFRET which could detect as small as 15 pg of methylated DNA from large alleles (Bailey et al., 2009). In this method, bisulfitite treated DNA was amplified with biotin tagged forward primers and fluorophore labeled reverse primer. Due to the strong streptavidin- biotin interaction, the labeled-PCR product was captured by streptavidin functionalized QDs. Then FRET stimulation between the QD donor and the fluorophore acceptor resulted the emissions of fluorophores (accompanied by quenching of QDs) which gave the relative level of DNA methylation. Although, this method was useful for multiplexed analysis and long-time monitoring of the methylation, it was also limited by high background noise and non-specific PCR amplicons. These drawbacks could easily be avoided using FRET linker probes (FLPs) as outlined by Keeley et al. (2012). In FLPs based assay, after bisulfitite conversion and PCR amplification with modified primers, the FLP distinguished the target amplicon, and thus eliminated the background noise from the non-specific PCR amplicons.

Ma et al., (2015) reported an advanced QD-FRET based DNA methylation assay, where enzymatically digested and PCR-amplified DNA was incorporated with a fluorophore to get the fluorescence readout. Signal amplification from QDs to the fluorophores upon interaction with FRET allowed quantitative determination of methylated DNA (unmethylated DNA is completely insensitive to FRET). More recently, this method has been extended to quantify DNA methylation in specimens derived from lung cancer patients (Ma et al., 2016). The advantages of using QDs in methylation assays lie in their inherent sensitivity and multiplexing capacity as well as their versatile ability in detecting methylated targets via other readouts. For example, QDs based assays have been well adapted in an electrochemical detection, known as methylation-specific ligase-detection reaction (EmsLDR), where multiple QDs have been used for the simultaneous detection of methylation levels (Dai et al., 2013).

Upconversion nanoparticles (UCNPs, e.g., Ln3+, Ti4+, Ni2+) have been studied as ECL active luminophores. Among these, tris (2, 2’-bipyridyl) ruthenium(II) (Ru(bpy)32+) and its analogues along with co-reactants tri-n-propylamine (TPPA, and its alternatives) have been mostly used (Weid and Wang, 2011) as they offer relatively better stability, good water solubility, spatial and temporal controllability, and wide dynamic range while producing luminescence. Generally, in DNA methylation assays (Fig. 2), a methylation specific chemical reaction pathway is incorporated on the electrode surface which only occurs in the presence of methylated DNA. Thus, if 5mC is present this reaction occurs and excites the luminescence resulting ECL light that allows quantification of 5mC level present in the sample (Kurita et al., 2012; Zhao et al., 2015). Surprisingly, until 2012, there was no report on ECL based DNA methylation detection assays. This could be due to the fact that conventional intercalation or hybridization based DNA methylation assays are believed to be not suitable to selectively confirm that luminescence are derived only from the 5mC present in the target sample.

In 2012, Kurita et al. (2012) have reported that ECL can be used to measure the level of DNA methylation. In this method, they used acetylcholinesterase labeled anti 5mC antibody to selectively capture the methylated DNA. Thiocoline was then produced from enzymatic reaction between acetylcholine and acetylcholinesterase, which was adsorbed on gold surface by gold-thiol affinity bonding. Upon application of potential to the electrode, ECL emission from Ru(bpy)32+ luminophore gave the quantitative level of the methylation present in target DNA (Fig. 3B). This method was successfully challenged in 48,502 bp-long DNA bacteriophage. A similar ECL method for the detection of DNA methylation and methyltransferase activity has been described by Li et al. (2012) where gold electrode was modified with the thiol-linked single strand DNA (ss-DNA) and ruthenium complex. Upon hybridization with the complementary ssDNA, this was treated with HpaII endonuclease. When cytosine was methylated, HpaII could not cleave the cytosine region whereas it cut between C-C in unmethylated cytosines, resulting a decrease in ECL signal.

Click chemistry based ECL assay has been developed to quantify DNA methylation (Zhao et al., 2015), where GO/AuNPs/luminol composite offers an extra specificity by producing amplified-ECL signal. This composite was functionalized with alkene to facilitate click chemistry. First azide-terminated duplex DNA modified electrode was formed upon hybridization of azide-terminated complementary DNA. Alkynyl GO/AuNPs/luminol composite was then accumulated on the electrode via click chemistry. When methylation-responsive restriction endonuclease DpnI was added on the electrode, GO/AuNPs/luminol was released from the electrode providing decreased ECL signal thereby confirming the presence of methylation. Unlike bisulfitite sequencing and restriction enzyme based methodologies, the ECL based methylation assays avoid the use of bisulfitite conversion, PCR amplification and enzymatic digestion steps. Other advantages of ECL based methylation assays include high sensitivity, rapid analysis, low volume of sample, and wide dynamic range. However, development of ECL high-throughput assay based on point-of-care testing with high sensitivity and good stability are required for routine clinical screening of DNA methylation. Though the production of multiple emitters in a common co-reactant has already been developed (Doeven et al., 2015) for multiplexed ECL detection of biological targets, it is yet to be incorporated in DNA methylation analysis.

3.2. Techniques based on electrochemiluminescence

In electrochemiluminescence detection system, oxidizing or reducing potentials are applied to chemical systems (containing target) in presence of luminophores. Depending on the presence of target, underlying chemical reaction is triggered causing emission of detectable lights from the luminophores (Liu et al., 2007; Wei and Wang, 2011; Doeven et al., 2015; Liu et al., 2015b). A number of materials (e.g., transition metal complexes, organic molecules and nanomaterials) have been studied as ECL active luminophores. Among these, tris (2, 2’-bipyridyl) ruthenium(II) (Ru(bpy)32+) and its analogues along with co-reactants tri-n-propylamine (TPPA, and its alternatives) have been mostly used (Weid and Wang, 2011) as they offer relatively better stability, good water solubility, spatial and temporal controllability, and wide dynamic range while producing luminescence.
3.3. Surface-enhanced Raman scattering (SERS) based techniques

Recent demonstrations have shown that SERS can be used in biosensing by two different ways: (i) the label-free detection of targets (direct detection of SERS fingerprint of adsorbed targets) via measuring the SERS intensity and (ii) indirect detection of the target species using SERS labels such as enzymes, dyes, quantum dots and fluorophores (Doering et al., 2007; Schlucker, 2009). It has also demonstrated that the target species do not always need to be directly adsorbed onto the metallic surface for obtaining the SERS spectra (Laing et al., 2016). To observe the most intense SERS response, target molecules must be within few nanometers distance from the metal surface (the degree of SERS signal enhancement is dependent on the distance between metal surface and the target, Laing et al., 2016).

Using these distinct SERS characteristics, DNA methylation can be assessed by immobilizing methylation specific capture probe on the metallic substrate of SERS along with the use of methylation specific SERS labels (Wang et al., 2015; Hu and Zhang, 2012). In recent years, SERS based readout has successfully been used in developing methylation analysis methods. For instance, Hu and Zhang (2012) developed a highly sensitive readout method using SERS labels which could detect 1% methylation level in p16 tumor suppressor gene. In this study, gold nanoparticle (modified with capture probe) was used as a SERS substrate and c5-dGTP, a guanine base with c5 label (cyamine dye), was used as a SERS label. When methylated DNA was attached to the gold nanoparticle-modified capture probe, it triggered a single base extension reaction between gold substrate and c5-dGTP resulting their hybridization. For an unmethylated DNA sample, no base extension reaction occurred. This binding event showed a high SERS readout with a detection limit of 3 pM DNA. In another approach, Wang and colleagues have enhanced the sensitivity of the SERS assay by coupling a ligase chain reaction (LCR) amplification with a SERS methylation assays (Wang et al., 2015). In their assay, bisulfite converted DNA was first amplified with PCR and then with LCR. In unmethylated LCR products, cytosines converted to thymines whereas in methylated LCR products cytosines remained unchanged. Following adsorption of LCR products on the SERS surface, specific Raman peaks were observed for methylated and unmethylated DNA. The method was successfully tested in a panel of breast cancer cell lines. More recently, this study has been extended to detect global DNA methylation using SERS nanotags and MBD. This advanced method is able to distinguish as minimum as 6.25% methylation changes in genomic DNA (Wang et al., 2016).

While classical Raman scattering is unable to detect molecular sample at low molar concentration (or low sample volume), SERS can successfully detect these targets with excellent sensitivity. The accuracy of the SERS detection lies on its unique electromagnetic enhancement mechanism, which is absent in conventional Raman and fluorescence readouts (Stiles et al., 2008). Additionally, compared to other available methods for DNA methylation analysis including conventional fluorescence, chemiluminescent and electrochemical approaches, SERS based DNA methylation assays have distinct advantages. In various fluorescence based assays, fluorophores usually suffer from large spectral overlap when the sample contain multiple targets thus making the detection difficult. This issue can easily be avoided by SERS as it provides sharp and narrow spectra allowing multiplexed analysis of the target (Harper et al., 2013; Laing et al., 2016). Although, it has been reported that SERS based detection of oligonucleotide is relatively more sensitive than conventional fluorescence based readout method, no comparative studies between SERS and fluorescence based DNA methylation detection have been reported. SERS based methylation assays require minimal sample preparation and avoid issues with interference, long term stability and non-specific adsorption which are common in conventional electrochemical sensing systems (Wang et al., 2008).

3.4. Techniques based on SPR

SPR can analyze the binding event of target molecules (in the solution) on the metallic surface (a thin metal film substrate) upon adsorption. If target binds the metallic sensor surface, refractive index of the metal substrate changes in real time. Thus, the amount of bound target and rate of the association and disassociation of targets can be calculated from the respective spectral shift (McDonnell, 2001; Homola, 2008). In SPR based methylation assays, the detection can be obtained by the direct detection mode where the methylation specific capture probe is immobilized on the SPR sensor surface. Methylated target in solution binds to the surface-bound capture probe, resulting a refractive index change detected by the SPR sensor. Methylation specific recognition elements (e.g., MBD protein or 5mC antibody) can also be used to further recognize the methylation sites (resulting a further refractive index change). This has been demonstrated by Pan et al. (2010), where a double recognition mechanism was used to enhance the analytical performance of the methylation assay. A complementary target DNA was first captured by the methylation specific probe immobilized onto the SPR sensor chip, which was further recognized by the recombiant MBD protein. Interaction between the methylated DNA and the MBD protein on the chip surface resulted in an increase in the refractive index, which generated a detectable optical signal. This method was capable of detecting 5 pM of methylated adenosomatous polyposis coli (APC) promoter gene.

The level of 5mC can also be detected without the use of methylation specific capture probe, MBD protein, or 5mC antibody, where methylated DNA can directly be adsorbed on SPR sensor chip (e.g., gold surface) resulting a refractive index changes compared to that of the unmethylated control. This has been experimentally demonstrated by Sina et al. (2014). The underlying principle of their method relies on the base dependent interaction affinity interaction between individual DNA bases and unmodified gold substrate (Sina et al., 2014; Koo et al., 2014; Koo et al., 2015). Bisulfite treatment converts unmethylated cytosines into uracils but methylated cytosine remains unchanged. A subsequent asymmetric PCR step amplifies the antisense strand of the bisulfite-treated DNA to generate ssDNA products. This step also converts uracils into adenines in the antisense strand of unmethylated DNA whereas guanines are generated in the methylated DNA. Since DNA–gold affinity interaction follows the trend A > C > G > T, the adenine-enriched unmethylated DNA leads to a larger level of adsorbed DNA on the SPR sensor surface in comparison to the guanine-enriched methylated DNA. This different adsorption patterns resulted two different spectral shifts for methylated and unmethylated sample allowing the methylation detection (Fig. 4). The sensitivity of this method was tested in genomic DNA extracted from breast cancer cell lines such as MCF7 and MDA-MB-231.

In recent years, several other DNA methylation methods have been developed based on SPR readout. For instance, an end-to-end nanorod assembly enhanced-SPR system has been developed for the detection of both DNA methylation and adenine methylation methyltransferase (DamMTase) activity (Li et al., 2015). The method showed a good relationship between the SPR spectral shifts. SPR readout has also been successfully adapted in microfluidics (Kurita et al., 2015) and molecular inversion probe (MIPs) (Carrascosa et al., 2014) based methylation analysis methods. For example, Kurita et al. (2015) have developed an SPR based microfluidic DNA methylation assay, where sequence-selective immunochemical discrimination of the 5mC status in a genomic sample has been detected within 45 min. This method avoids the use of conventional bisulfite treatment, PCR amplification, sequencing and enzymatic digestion steps. In the MIP based SPR assay (Carrascosa et al., 2014), bisulfite treated genomic DNA was investigated with a MIP using a fill-in approach (Palamisamy et al., 2011), where MIP binds to the DNA and generates a wide gap of several bases between the MIP recognition ends. This represents the methylated DNA.
region to be investigated. This gap was then filled by a polymerase and subsequently ligated by a ligase, leading to a circularized DNA probe carrying the imprinted information from the target region. Finally, the MIP circularized product was amplified by asymmetric PCR to generate a ssDNA amplicon, which was captured by a receptor oligo sequence previously immobilized onto the SPR sensing surface. This method was successfully applied to detect regional methylation in the cancer cell lines.

Besides the real-time and label-free nature of the SPR readout, it has other advantages. For example, (i) offers the analysis of receptor–target interactions with a wide range of molecular weights and affinities, (ii) it is also highly compatible with different chemical environments (e.g., temperature, ionic strength and pH) (McDonnell, 2001), and (iii) it can explain the DNA molecular recognition events via analyzing binding kinetics and thermodynamics under different conditions. For example, in a microarray based DNA methylation assay, SPR was used to measure the binding kinetics MBD protein and dsDNA (Yu et al., 2010). Although these current demonstrations offer real-time, label-free analysis of DNA methylation, they are limited by the poor sensitivity of SPR readout (Green et al., 2000).

3.5. Colorimetric detection techniques

Colorimetric assays for the detection of DNA methylation has gained recent interest as they are simple, reliable, cost effective, portable and targets can be detected with the naked eye. Since native nucleic acids do not show absorption in the visible region, various color reporting groups such as small organic dyes, conjugated polymers, enzymes coupled to a chromogen and metallic nanoparticles have been used to develop colorimetric biosensors (Liu, 2009). Among these groups, metallic (e.g., gold) nanoparticles functionalized with either a probe DNA or an aptamer for capturing the target analyte (Kanjanawarut and Su, 2009; Elghanian et al., 1997) have been commonly used to develop colorimetric biosensors. When target DNA contains complementary sequences of the probes, it causes particles' aggregation through sandwich hybridization that can be detected as color change of the colloidal solution. Another popular approach uses salt induced aggregation of gold nanoparticles to visualize the targets (driven by the London-van der Waals attractive force) (Sato et al., 2003; Li and Rothberg, 2004). These nanoparticle aggregation based assays have been used for naked eye detection of DNA methylation (Ge et al., 2012; Lin and Chang, 2013). In this assay, anti 5mC antibody modified with magnetic microspheres (MMPs) were selectively attached with the CpG region of APC (Ge et al., 2012). An APC specific probe was then added which captured the MMPs-APC complex. After the capture event, the hybridized complex was denatured by heating. The released capture probe was then mixed with unmodified gold nanoparticle (AuNP) and salt solutions for colorimetric detection. In this system, the presence of DNA methylation hinders the salt-induced aggregation of AuNP. In contrast, unmethylated DNA facilitates the aggregation, leading to a quick color change from red to purple. The method was sensitive to detect 80 fM of target DNA. Lin and Chang (2013) have developed another colorimetric assay for DNA methylation detection based on AuNPs aggregation. The applicability of the method has been tested to analyze methylation level in nasopharyngeal carcinoma cells lines.

To improve the sensitivity of the colorimetric assay, MBD protein based enrichment method can be adapted in methylation assay. For example, Wee and colleagues have developed a colorimetric assay for the rapid detection of both global and regional methylation, where
enzymatically digested-genomic DNA was labeled with biotin (via a fill-in reaction with Klenow polymerase and biotin-dNTPs (Fig 5) (Wee et al., 2015a). Magnetic beads functionalized-MBD were then selectively bound to the biotin conjugated methylated region. Next streptavidin coated horseradish peroxidase (SA-HRP) was used to recognize biotinylated methylated region. The methylation level was then visually evaluated via HRP/H₂O₂ system coupled to 3,3′,5,5′-tetramethylbenzidine. It is evident that unlike many other optical readout methods, colorimetric assays require less sophisticated optical setup to detect DNA methylation which can make on-site detection of DNA methylation more manageable.

4. Conclusion and perspectives

We have thoroughly reviewed the recent advances in optical detection strategies for DNA methylation. We have also addressed the major methodological shortcomings of these strategies. It is now evident that most of these strategies are not suitable for routine clinical applications as these methods typically rely on cumbersome chemical treatment (e.g., bisulfitation conversion), amplification (e.g., PCR) and enzymatic digestion of target samples. We believe that the most suitable method for diagnostics or clinical applications should not be too much technically complicated. In our opinion the method with the ability of detecting DNA methylation directly with an inexpensive detection tool will ultimately be the future of methylation based epigenetic research.

In optical detection strategies, development in laser, filters and other optical set up of these techniques can contribute to the development of more suitable commercial methylation biosensors. The ongoing research should try to resolve interference created from sample population variation. Researchers should also exploit different options to improve multiplexing capability of DNA methylation. Different sandwich model with various transduction steps can be designed in the sensors to increase the target response. Moreover, innovative approaches should be established to remove false negative result and nonspecific binding. It is obvious that a considerable amount of work needs to be performed to meet the remained challenges and to ensure further enhancement of the methylation biosensors for achieving desired level of accuracy and sensitivity. In our opinion, what have been achieved so far will serve as the foundation for the development of novel strategies for DNA methylation. We forefeel that in the near future, systematic research will translate many of these proof-of-concept ideas into the suitable point-of-care DNA methylation biosensors.

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