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DNA methylation analysis in human cancer

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Chapter summary

The functional impact of aberrant DNA methylation and the widespread alterations in DNA methylation in cancer development has led to the development of a variety of methods to characterize the DNA methylation patterns. This chapter will critique and describe the major approaches to analyzing DNA methylation.

DNA methylation: background and relevance

“The existence of phenotypic differences between cells with the same genotype merely indicates that the expressed specificities are not determined entirely by the DNA present in the cell—that other devices, epigenetic systems, regulate the expression of the genetically determined potentialities”¹

The plethora of cell types within an organism share a unifying genome. Despite this genetic unity, the various cell types, functions and phenotypes within an individual's cytochrome remain widely varied due to vast differences in their gene expression, both quantitatively and qualitatively. These variances are known to be dictated by so-called epigenetic mechanisms, such that individual cell types and developmental states, within an individual organism, have unique epigenomes. Thus, epigenetic mechanisms are crucially relevant in differentiation and development of all cell types, including those which do so in pathological contexts.

The field of epigenetics has grown exponentially since its originally proposed definitions, over 70 years ago², and is now a central and prominent research field. As the interest in epigenetics grows, so too does the awareness of the many heterogeneous situations in which epigenetic mechanisms are important pathologic facilitators. Epigenetics is now a focus of many medical disciplines ranging from autoimmunity to embryology and from psychiatry to oncology^{3–6}. The magnitude of epigenetics is daunting when one considers that it is a field which has not yet been explored in anywhere near as much depth as the field of genetics, and yet there exists a far wider range of normal and abnormal epigenetic marks compared to genetic alterations^{7, 8}. Indeed, methylation patterns are quite variable both within and between tumor types^{9, 10}.

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Epigenetic characterizations are ongoing across multiple disciplines, in both tissue and disease specific contexts, its study further diversified by the variety of different relevant mechanisms, which include DNA methylation, modifications to chromatin, loss of imprinting and non-coding RNA. The most relevant and most comprehensively considered epigenetic mechanism is DNA methylation^{11, 12}.

The term DNA methylation classically refers to the methylation of DNA cytosine residues at the carbon 5 position, and typically it specifically implies methylation of that cytosine in the relatively rare CpG (cytosine-phospho-guanine) dinucleotide context, and especially those restricted areas of the genome known as CpG islands, in which CpG sites are highly prevalent^{13–15}. Another recently discovered DNA methylation mark is 5-hydroxycytosine, described as the “6th base”. 5-hydroxycytosine is formed by the conversion of 5-methylcytosine by TET enzymes (methylcytosine hydroxylases^{16, 17}). 5-hydroxycytosine is detected in stem cells during in early development¹⁷. Mutations in IDH genes alter production of alpha-ketoglutarate (alpha-KG) and 2-hydroxyglutarate (2-HG) and these alterations inhibit TET enzyme activity and influence 5-ohmC¹⁸. Bisulfite modification cannot be used to detect 5-ohmC but another enzymatic modification¹⁹. In addition; other nucleotides are rarely methylated mainly in plants). Aberrant DNA methylation is often oncogenic, such that dense methylation of CpG islands in promoter regions generally has a repressive effect on transcription, and conversely, a loss of methylation in certain CpG islands have been shown to facilitate transcription^{20, 21}. This basic paradigm does not always apply however. Hypomethylation of a repressor locus can result in an increase in transcription (e.g. for hTERT)²². DNMT3a and DNMT3b perform *de novo* DNA methylation of unmethylated CpG dinucleotides^{23–26} while DNMT1 maintains DNA methylation during replication^{23, 27}. CpG methylation usually occurs on both DNA strands, which further ensures replication, making this a heritable modification²⁸.

In-depth knowledge of the patterns of DNA methylation in human cancers is clearly crucial to better understand disease pathogenesis and to identify therapeutic targets and tumor markers. Comprehensive characterization of DNA methylation patterns within normal and abnormal cells is currently underway, in the context of the Human Epigenome Project^{29–31}.

DNA methylation analysis

Multiple advancements in sequencing and microarray technologies have enabled the development of many tools for analyzing DNA methylation. These novel strategies include all relevant steps in DNA methylation analysis, and permit the use of custom strategic combinations^{12, 32–36}.

Most methods evaluate DNA methylation, beginning with a **methylation specific pre-treatment step**, followed by **polymerase chain reaction (PCR)**, concluding with detection of **methylation patterns**. Within any individual experiment, the final choice of method will be dictated by the desired depth of coverage, throughput, run time, sample DNA volume and quality, a desire for quantitative versus qualitative methylation information, and the preferred forms of bias.

This chapter will describe the crucial pre-treatment steps required for DNA methylation analysis, and some of the downstream detection methods. It will then discuss some of the more useful techniques in terms of cost-effectiveness, depth of coverage and reliability.

Methylation Specific Pre-Treatment

Most DNA methylation analysis methods require the employment of polymerase chain reaction (PCR) to provide sufficient DNA for detection of methylation patterns. PCR is used

extensively in DNA methylation analysis; however PCR does not include DNA methyltransferase steps so PCR products do not contain original template DNA methylation marks. To avoid the loss of methylation information with molecular biology techniques such as PCR and cloning, pre-treatment of DNA is required to identify methylated CpGs^{34, 35}. The technical options available for pre-treatment are bisulfite modification, methylation-specific endonuclease digestion and affinity enrichment with either methylation-specific immunoprecipitation or methylation-specific protein hybridization.

Bisulfite modification

Bisulfite modification is the most widely used of all the pre-treatment options available for DNA methylation analysis. The technique was first described by Frommer over two decades ago³⁷, and is central to the majority of DNA methylation analytical strategies. Bisulfite modifies unmethylated cytosines converting them to uracils, while 5-methylcytosine residues remain unmodified. Uracil residues then become thymine residues during any subsequent PCR amplification of bisulfite-modified DNA, whereas the originally methylated cytosines become unmethylated cytosine residues during PCR amplification. From this permanently changed sequence, one can infer that any remaining cytosine in the amplicon was methylated in the original DNA sequence. The bisulfite-modified DNA can then be analyzed further with a variety of methods, from the very low-scale and low-cost, methylation specific PCR (MSP), to endonuclease-digested fragment size comparison detection methods, to array based detection methods, and finally to analysis using next generation sequencing, which provides the most comprehensive and definitive methylation analysis available. DNA damage is an undesirable result of bisulfite treatment. With this treatment, the aerobic oxidation of sulfite generates free radicals³⁸, as well as promoting chain fission of double-stranded DNA³⁹. Certain optimization strategies have been suggested to improve the bisulfite-modification step, including limited presence of air and free radicals³⁹, as well as increasing concentrations of bisulfite to the order of 1M or higher, which both seem to improve DNA integrity⁴⁰.

Methylation-Specific Endonuclease Digestion

Endonuclease cleavage is another method used to delineate methylation sites, in a replicable way. This strategy involves cleavage of the DNA at methylation-sensitive (cleaves only at unmethylated CpG site, e.g. *HinP1I*, *HpaII* or *NotI*), methylation-dependent (cleaves only at methylated CpG, e.g. *McrBC*) or methylation-insensitive (cleaves at either methylated or unmethylated CpG sites, e.g. *MspI*) sites. This method generates fragments of DNA of differing length, determined by methylation status, which can then be comparatively analyzed by fragment size analysis (gel electrophoresis, high-resolution melting (HRM) or high performance liquid chromatography (HPLC)), on an array based platform, or using sequencing methods. Endonuclease-mediated enrichment of methylated regions is effective for generating both qualitative and quantitative methylation information, and despite the requirement for DNA of high integrity, PCR adaptations of these techniques can reduce reliance on DNA input quantity⁴¹. This restriction technique, by definition, will introduce recognition site biases as well as providing a relatively poor resolution, only testing the few CG dinucleotides located within that restriction site. The analyses of these fragments are therefore not wholly comprehensive, and additionally, incomplete digestion can lead to incorrect calls, which a problem that can be limited by a sequencing step. A follow-up affinity enrichment step has also been proposed as a way to mitigate this problem⁴². Coverage and resolution of DNA methylation analysis, with endonuclease enrichment, is a function of distribution of the recognition sequences of the endonuclease in use. The endonuclease-based strategy can incorporate endonuclease combinations which help to overcome restriction-site related biases.

For example using the isoschizomers *HpaII* and *MspI* (used in the combination technique HEIP), one can assess CpG sites at nearly 99% of CpG islands⁴¹. This combination samples more CpGs than the isoschizomers *SmaI* and *XmaI* combination which is used in the methylated CpG island amplification (MCA) technique, but the latter combination provides a more specific restriction⁴³. Alternatively, if one opts to use a relatively non-specific endonuclease such as *McrBC*, one will delineate virtually all methylated CpG islands, however this strategy, in turn, is limited by the requirement of two methylated cytosines to be within a relatively short distance of each other.

After methylation restriction, subsequent analysis often involves array-based detection, but as the cost of sequencing improves this will become the most satisfactory approach. Among other advantages, sequencing allows for discovery beyond the limitations to novel discovery when using array-based techniques⁴⁴. Indeed, to further complicate matters, some pre-treatment strategies involve integration of restriction digestion with bisulfite treatment, such as in Combined Bisulfite Restriction Analysis (COBRA), whereby bisulfite modified DNA is then restricted in a methylation specific way, and in the mirrored approach, reduced representation bisulfite sequencing (RRBS), which involves methylation specific restriction preceding bisulfite modification and sequencing.

Affinity Enrichment

There are two relevant affinity enrichment techniques used to enrich methylated DNA fragments, for subsequent analyses and comparisons between groups. They both involve random (methylation non-specific) shearing of DNA, followed by application of either an antibody (**anti-5-methyl-cytosine**), or a recombinant protein (**methylation-specific binding protein**) which enrich the fragments of interest and permit a more extensive analysis. The technique requires a sufficient input quantity, to promote the efficiency of enrichment; however, DNA integrity relatively is not as crucial, in this technique.

Methylation-Specific Immunoprecipitation

Methylation specific immunoprecipitation is one of the two forms of affinity enrichment. This treatment, directed towards randomly fragmented DNA, involves the immunoprecipitation of 5-methyl cytosine residues with an anti-5-methyl cytosine antibody, such that fragments containing these residues are retained (on beads, with the MeDIP protocol⁴⁵), while unbound fragments are discarded. The DNA is fragmented, often by sonication, to avoid biases associated with fragmentation by endonucleases. The resulting enriched pool of methylated DNA fragments is subsequently processed to remove bound antibodies and from this point can be examined with various methylation detection platforms. The research question will determine the detection platform chosen. It is possible to analyze the sample to sample DNA methylation differences in a relatively quantitative way, for example by high resolution melting (HRM), by high performance liquid chromatography (HPLC), or indeed one can use higher-throughput platforms such as array-hybridization (e.g. MeDIP⁴⁵)⁴⁶ or finally, sequencing technologies²¹, which give qualitative as well as quantitative information.

The fragments enriched by this method can, however, represent an overestimation of methylation levels due to the extreme specificity of the antibody, such that fragments containing as low as one methylated cytosine within a sequence are detected and may often misrepresent otherwise sporadic and insignificantly methylated nucleotides⁴⁶. This bias renders immunoprecipitation to be the least preferable of the two affinity enrichment techniques, which can be somewhat mitigated by presence of control samples of both unmethylated and methylated DNA, allowing a more calibrated correlation between immunoprecipitated samples and methylation status.

Methyl-binding protein capture

This technique involves the random shearing of genomic DNA, that is then precipitated by recombinant methyl binding domains which have affinity for methylated genomic DNA⁴⁷, either within a column⁴⁷, on a microarray⁴⁸ or immobilized on magnetic beads⁴⁹. As with the methylated DNA immunoprecipitation, the captured fragments are subsequently removed from the binding proteins, which then are freely amenable to various applications, including PCR and sequencing. The fragment pool generated by this technique is somewhat more relevant than that generated by immunoprecipitation, as MBD binds with preferential affinity to methylated cytosines in close proximity, and so precipitated fragments contain more biologically relevant methylation patterns than the fragments containing sporadically methylated CpGs, which are frequently precipitated with antibodies, and which likely have less biological relevance⁵⁰. The coverage and resolution of the DNA methylation information following this protocol are largely determined by which detection platform is selected, ranging again from gross relative quantitative analyses such as high-resolution melting (HRM), gene-specific PCR^{49, 51}, to high-throughput patterns analyses such as microarrays, or analysis as comprehensive as massively parallel sequencing (e.g MethylCap-seq, or MIRA-seq^{52, 53})⁵⁴.

Materials for Methylation-Specific Pretreatments

Bisulfite Modification⁵⁵

1. Isolated genomic DNA
2. Freshly prepared 3M NaOH solution
3. Freshly prepared, saturated sodium metabisulfite (pH 5.0)
4. 10 mM quinol
5. Mineral oil
6. Wizard DNA Clean-up System (Promega)
7. 80% isopropanol
8. 5M ammonium acetate (pH 7.0)
9. tRNA
10. 100% ethanol

Restriction Enzyme Digestion⁵⁶

1. Isolated, high quality genomic DNA
2. Endonuclease(s) of choice
3. Enzyme buffer (provided with enzyme)
4. 1% agarose gel
5. TE (pH 8.0)
6. Saturated phenol:chloroform mix (1:1)
7. Glycogen
8. 3M NaOAc (pH 5.2)
9. 100% ethanol
10. 10mM Tris (pH 8.0)

11. Pre-annealed linker adaptor mixture
12. T4 DNA ligase buffer (Invitrogen, Carlsbad, CA)
13. T4 DNA ligase (Invitrogen, Carlsbad, CA)
14. 10mM Tris-HCl (pH 8.0)

Methyl-Specific Immunoprecipitation^{57, 58}

1. Isolated genomic DNA
2. 5-Methylcytidine antibody (Abcam)
3. IP Buffer: 10 mM NaPO₄ (pH 7.0), 140 mM NaCl, 0.05% Triton X-100.
4. Dynabeads® Magnetic Beads (Life Technologies)
5. DynaMag™ magnets (Life Technologies)
6. Proteinase K
7. Digestion buffer: 10 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS, 50 mM NaCl
8. 1:1 phenol/chloroform (pH 7.0)
9. 3 M sodium acetate
10. Glycogen
11. Ethanol 70%
12. Ethanol 100%

Methyl-Binding Capture

1. Isolated genomic DNA
2. MBD-binding protein
3. 0.25M NaCl
4. 20mM Tris (pH 8.5)
5. Ni-NTA-agarose beads (Qiagen)
6. 5M guanidine HCl
7. 30% isopropanol
8. 20 mM HEPES
9. 5 mM EDTA (pH 7.5)
10. 1:1 phenol/chloroform (pH 7)
11. 3 M sodium acetate
12. Glycogen
13. 70% ethanol
14. 100% ethanol

Methods for Methylation Specific Pre-Treatments

Bisulfite Modification⁵⁵

Briefly, DNA is denatured and the reaction of bisulfite with cytosine gives a cytosine sulphonate derivative. Deamination of the resulting cytosine–bisulfite derivative results in a uracil sulphonate derivative, uracil is formed by subsequent removal of the sulphonate group by an alkali treatment. Bisulfite does not have this effect on methylated cytosine.

Denaturation of Genomic DNA—(See note 1)

1. Incubate samples at 37 °C with freshly prepared 3M NaOH for 15 minutes, followed by a 2 minute incubation at 90 °C and then immediate placement on ice for 5 minutes, which will ensure the DNA remains denatured.

Sulphonation and Hydrolytic Deamination—(See note 2)

- 2 Centrifuge sample at 10,000g and 4 °C for ~10 seconds and immediately add sodium metabisulfite, quinol and mineral oil.
- 3 Mix solution gently and centrifuge for 10 seconds
- 4 Incubate solution at 55 °C, for 4–16 hours.

(See note 3)

- 5 Remove the DNA solution from beneath mineral oil.

Desalting and Alkali Desulphonation—(See note 4)

- 6 Purify DNA using Wizard DNA Clean-up System (Promega), as per manufacturer instructions.
- 7 Desulphonate the purified DNA by incubating with 3 M NaOH at 37 °C for 15 minutes.
- 8 Centrifuge briefly and add tRNA (10 mg ml⁻¹).
- 9 Add 5 M ammonium acetate at pH (7.0).
- 10 Add ice cold (–20 °C) 100% ethanol.
- 11 Mix well by inversion and leave at –20 °C for an hour or longer to precipitate the DNA.
- 12 Centrifuge at 14,000g for 15 minutes at 4 °C.
- 13 Remove all supernatant and air dry for 20 minutes.
- 14 Resuspend the precipitated DNA in DNA buffer or H₂O, occasionally vortexing at room temperature for ~2 hours, followed by PCR use or storage.

(See note 5)

¹DNA that is of high molecular weight should be fragmented prior to use, to ensure complete denaturation. This can be achieved with sonication.

²The reaction should take place in the dark, or with foil around the sample, to prevent oxidation

³Length of incubation will vary depending on DNA amount. Degraded tissue such as that isolated from paraffin embedded tissue will require a shorter incubation.

⁴Desalting removes unwanted bisulfite ions, and the alkali step will ensure successful completion of bisulfite conversion

⁵DNA may be stored in this way at –20 °C for up to 10 years, and can be subsequently re-precipitated and used in PCR.

Restriction Enzyme Digestion⁵⁶

Fragmentation

1. Incubate intact, high molecular weight genomic DNA with the methylation-specific endonuclease(s) of choice and with manufacturer recommended buffer, overnight at 37°C.
2. Verify appropriate fragmentation by running on 1% agarose gel.

Purification

- 3 Add TE (pH 8.0) and phenol:chloroform mix to the digested DNA and vortex briefly.
- 4 Centrifuge this solution at 16,000g for 10 minutes at room temperature, and collect aqueous phase.
- 5 Add glycogen and 3M NaOAc pH (5.2), and mix well.
- 6 Add 100% ethanol, vortex and centrifuge at 16,000g for 45 minutes at 4°C
- 7 Remove supernatant and wash pellet with 70% ethanol
- 8 Remove all traces of ethanol and resuspend pellet in 10mM Tris pH (8.0)

Linker ligation—(See note 6)

- 9 In a PCR tube combine digested DNA and the pre-annealed linkers
- 10 Place this reaction in a thermocycler at 55°C for 5 minutes, then decrease to 4°C over one hour. Add T4 ligase into the reaction mix once reaction reaches 4°C.
- 11 Incubate this solution at 16°C overnight.
- 12 Dilute this product with 10mM Tris-HCl (pH 8.0), and continue to PCR or store indefinitely at -20°C.

Methyl-Specific Affinity Enrichment Methylated DNA Immunoprecipitation^{57, 58}

(See note 7)

Sonication of Genomic DNA—(See note 8)

1. Place isolated genomic DNA in Bioruptor (Diagenode). Bioruptor should be at 4°C.
2. Sonicate on highest setting for ~four 15 minute cycles of 30 second on-off intervals.
3. Centrifuge the DNA after each cycle and cool the sonicator for 10 minutes after each cycle, by filling with ice.
4. Validate DNA fragment size range and concentration with the Agilent Bioanalyzer 2100.

⁶The fragmented DNA will have single-stranded overhangs that may degrade, so linker ligation should be performed on same day.

⁷DNA sonication and the MeDIP protocol must be performed in siliconized tubes

⁸Duration of sonication depends on starting DNA molecular weight. High molecular weight DNA such as from cell lines will require more sonication than archival, degraded DNA. The ideal fragment size is 300–1000 base pairs.

Immunoprecipitation of DNA

- 5 Place fragmented DNA in a siliconized centrifuge tube, and denature at 95°C for 10 minutes.
- 6 Cool immediately and completely (~5 minutes).
- 7 Add monoclonal antibody and IP buffer and incubate this solution at 4°C for 2 hours in a rotating tube holder.
- 8 Add washed Dynabeads® to the IP reaction for 2 hours, maintained at 4°C.
- 9 After incubation, wash beads at room temperature, add digestion buffer and treat the reaction with Proteinase K overnight at 50°C.

Purification

- 10 Add 1:1 phenol/chloroform (pH 7) and vortex thoroughly
- 11 Centrifuge for 10 minutes at 13 000 g.
- 12 Collect aqueous fraction, add 3 M sodium acetate and vortex.
- 13 Add glycogen and vortex
- 14 Add 100% ethanol, vortex, and incubate at -20°C for 20 minutes
- 15 Centrifuge at 13 000 g for 20 minutes at 4°C
- 16 Remove ethanol
- 17 Add cold 70% ethanol to wash. Vortex briefly, and centrifuge at 13 000 g for 20 minutes at 4°C.
- 18 Remove ethanol
- 19 Air dry the pellet for 10 minutes and resuspend in sterile dH₂O
- 20 This DNA product is now amenable to PCR.

Methylation Binding Domain Precipitation

Sonication of Genomic DNA—(See note 8)

- 1 Place isolated genomic DNA in Bioruptor (Diagenode). Bioruptor should be at 4°C.
- 2 Sonicate on highest setting for ~four 15 minute cycles of 30 second on-off intervals.
- 3 Centrifuge the DNA after each cycle and cool the sonicator for 10 minutes after each cycle, by filling with ice.
- 4 Validate DNA fragment size range and concentration with the Agilent Bioanalyzer 2100.

Methyl-Capture

- 5 Add sheared DNA to the MBD-binding protein and add a buffer of 0.25M NaCl, 20 mM Tris (pH 8.5) to adjust volume, and rotate for 30 minutes.
- 6 Add 50% solution of Ni-NTA-agarose beads and rotate for 30 minutes.
- 7 Wash the beads four times with the buffer containing 0.25M NaCl, 20 mM Tris (pH 8.5).

- 8 Add 5M guanidine HCl, 30% isopropanol, 20 mM HEPES, 5 mM EDTA (pH 7.5) to the beads and incubate for 10 minutes, collecting the supernatant.

Purification

- 9 Add 1:1 phenol/chloroform (pH 7) and vortex thoroughly
- 10 Centrifuge for 10 minutes at 13 000 g.
- 11 Collect aqueous fraction, add 3 M sodium acetate and vortex.
- 12 Add glycogen and vortex
- 13 Add 100% ethanol, vortex, and incubate at -20°C for 20 minutes
- 14 Centrifuge at 13 000 g for 20 minutes at 4°C
- 15 Remove ethanol
- 16 Add cold 70% ethanol to wash. Vortex briefly, and centrifuge at 13 000 g for 20 minutes at 4°C .
- 17 Remove ethanol
- 18 Air dry the pellet for 10 minutes and resuspend in sterile dH_2O
- 19 This DNA product is now amenable to PCR.

Analysis of Differential Methylation Patterns

DNA methylation can be characterized in terms of methylated sequence patterns on specific individual molecules, or characterized averagely (and more crudely) at specific loci but across many DNA molecules³²

Most DNA methylation detection strategies have some bias; hence it is common to confirm DNA methylation alterations with additional techniques. The high DNA quantity and quality requirements of many techniques make cell lines an appealing sample cohort, as opposed to primary tissues, and can be a useful as standardized sample cohorts in the clinical setting.

Primer Specific Detection

Methylation specific PCR (MSP) and Methylation Sensitive Nucleotide Primer Extension (Ms-SNuPE) are two primer mediated detection strategies which require the use of bisulfite modified DNA, where the presence or absence of PCR product indicates the methylation status of the locus under consideration. Differential amplification of unmethylated vs. methylated templates in MSP is a limitation^{59, 60, 37, 61–63} MSP assays may also employ methylation specific fluorescent probes for real-time quantification (e.g Fluorescent conjugated polymer-based (FRET) and MethyLight^{64–66}).

High Resolution Melt Curve analysis (HRM)

The melting profiles of bisulfite-modified PCR products can be used to indicate methylation status, when unknown samples are compared with both unmethylated and methylated controls. This involves a bisulfite pre-treatment, followed by unbiased PCR amplification of both methylated and unmethylated templates of the regions of interest. Specific protocols such as methylation-sensitive HRM (MS-HRM) provide an in-tube PCR and melting analysis platform, enhancing labor efficiency of this sensitive technique⁶³. A visual platform for this technique integrates a fluorometer coupled thermal cycler, which, by virtue of SYBR Green I fluorescing when bound to dsDNA, exhibits a decrease in fluorescence when melting point is reached⁶⁷. Melting analyses are a crude measure of methylation levels,

which can distinguish partial and full methylation, but cannot provide detail of the order of single cytosines. This detection technique is applicable in the process of selecting regions of interest to pursue on a sequencing platform.

DNA microarrays

Technologies involving microarray manufacture have progressed greatly in recent years. Array hybridization analyses have moved beyond simply characterizing gene expression, and are no longer confined to interrogating annotated regions. Advanced chip customization tools now enable array high throughput analysis exquisite coverage and resolution, such that the most advanced microarrays, known as tiling arrays, can render information regarding whole genomes, and of the order of single bases⁶⁸. Hybridization signals remain predictions with these platforms. However, when coupled with appropriate confirmatory experiments such as RT-PCR amplification, cloning of cDNA and sequencing, custom arrays have potential to generate novel discoveries.

The progress seen in custom microarray manufacture has sparked an evolution within DNA methylation analysis, and the technique has matured beyond its previously limited utilities⁶⁹. Array platforms have been adapted to accommodate all forms of methylation-specific pre-treated DNA, such that individual platforms exist for bisulfite modified DNA, for methylation-specific endonuclease digested DNA, and for methylation affinity enriched DNA pools. Oligonucleotide methylation analysis arrays can thus be differentiated by probe size and coverage, as well as input DNA format.

Previously, bisulfite sequencing had been the only method by which to determine novel methylated sequence, whilst all other methods were limited to presence/absence interrogations of known sequences. Although bisulfite sequencing remains the gold standard for determining methylated sequence patterns, the new potential of tiling arrays equates to potential for drastically more cost effective methods of querying novel DNA samples, with the additional advantage of much higher throughput. Previously, array hybridization of bisulfite modified DNA involved the adaptation of arrays designed for genomic interrogations, which meant overall hybridization was invariably low with this adaptation, due to the reduced complexity of the bisulfite modified sequence. Custom array manufacture now allows for modified oligonucleotide probes which may be designed to account for the decreased sequence complexity seen with bisulfite modification.

Bisulfite modified DNA can be analyzed by oligonucleotide arrays in an intuitive way, whereby methylated vs. unmethylated sequences will generate differential hybridization signals, depending whether or not they successfully bind a methylation-specific probe. One can adapt these custom arrays to encompass comprehensive sequence, as seen in the Infinium platform, by Illumina, which requires a whole genome amplification of the bisulfite modified sequence, followed by fragmentation and hybridization. With the Infinium platform each CpG site is interrogated with a methylation specific, allele-specific and multiplex approach, in the context of a bead-based array platform⁷⁰. Alternatively, it is possible to focus on more specified CpG sites of interest, as in the less sequence-extensive GoldenGate bead array platform, also by Illumina, which is still a high-throughput single-nucleotide polymorphism (SNP)-genotyping bead-based array platform, which incorporates allele-specific extension⁷¹, but which instead relies on both methylated and unmethylated primer extension of bisulfite-modified regions of interest, such that the differential PCR products of either methylation status are labeled with distinct fluorescent dyes⁷², and so provide a means of differentiating those which hybridize to the bead representing the CpG site of interest. This GoldenGate platform still provides exquisite detail but only represents limited CpG sites⁷¹. The multiplex and allele specific format of these chips allow for

appropriately detailed quantification scales, and make them appealing in the context of clinical applications, such as prognostication.

Methylation-specific endonuclease digestion of DNA generates fragments which are often carried through to array based analytical platforms. These different array platforms vary widely, based on endonuclease(s) used (methylation-sensitive, methylation-dependent, or both). Indeed, approaches additionally may incorporate Differential Methylation Hybridization (DMH) strategies, which may employ various endonucleases to generate a desirable pool of fragments for parallel comparisons^{73, 74}. Strength of data arising from these array hybridization analyses vary largely based on this control DNA fragment pool parameter, as sub-optimal restrictions can generate incorrect calls, and so a well-generated control-restricted pool of DNA can largely impact validity.

Specific variations of DMH exist, depending on methylation-specific endonuclease(s) used. For example, the method “Methylscope” analyzes previously randomly digested DNA a second time using the methylation-dependent endonuclease (*McrBC*). Microarray-based methylation assessment of single samples (MMAss) involves digesting and comparing hybridization patterns of two DNA pools of a single sample differentially with either a methylation-sensitive or methylation-dependent enzyme. These types of comparative restrictions facilitate recognition of arbitrarily restricted fragments, and help to focus on those sites which are of genuine biological relevance⁷⁵. Fragments containing the methylation status amenable to restriction are cleaved and thus no longer bind to probes specific for the sequence of interest.

As hybridization will indicate a lack of cleavage, one can infer the methylation statuses of the interrogated sequences through these comparisons. However, one cannot elucidate CpG specific information, but can only provide an indication of methylation status of a region, from which to pursue further analysis. The shorter the recognition probe is, the more reliable the results will be, as a cleaved fragment will be less likely to bind arbitrarily in this scenario. One can analyze DNA methylation patterns on a genome-wide scale, as with the HEIP assay⁴⁴, and the MCA coupled to CpG island microarray^{76, 77}. Specificities and resolution of endonuclease digested DNA methylation analyses are perhaps more reliant on design of endonuclease combinations used, and parallel control pools of standardized DNA fragments for comparison, rather than the specific microarray design, though all of these features should ideally be optimal.

Array hybridization of affinity enriched genomic fragments to tiling arrays is done often in conjunction with PCR. As affinity enrichment such as MeDIP often suffers from over-estimation of methylation, array hybridization can provide a mechanism of stratification of samples, to retain those which are methylated at various loci of interest for further validations⁷⁸. Methyl-binding domain (MBD) agarose chromatography additionally can fractionate and thus stratify DNA according to its extent of methylation, to be then hybridized to tiling microarrays to identify unmethylated, lightly methylated, and heavily methylated DNA fragments.

The cost effectiveness, and reduced labor associated with this platform renders it an ideal approach for analyzing large numbers of samples. Improvements in technology are moving beyond previous limitations such as ascertainment bias, and so it is an approach now which affords comparable esteem to sequencing approaches, without the price tag.

Methylome Sequencing

Rapid evolution and improvements in next-generation sequencing platforms are expected to permit whole genome sequencing for ~\$1000 in the very near future. Some of the latest

contributors to the new field of next-generation sequencing include developments in emulsion PCR, and the pyrosequencing and ion semiconductor sequencing platforms

Next generation sequencing technologies that enable accurate quantification of bisulfite sequences (methylated vs. unmethylated CpGs), are the gold standard in the context of methylation sequencing analyses^{79, 80}. The appeal of this technique lies largely in the coverage and single-base resolution of information generated, and the quantification allows for pertinent distinctions regarding allele-specific methylation, as well as distinguishing heavily or lightly methylated regions, which all help compose a biologically relevant picture.

Although single nucleotide sequencing for methylation changes is comprehensive, the cost, labor and bioinformatic load associated with sequencing entire genomes remain an obstacle. It is difficult to interpret the significance of single nucleotide methylation alterations across a whole genome, particularly when many thousands of alterations are present in a cancer sample.

Several optimization methods are available to counter the limitations to large sample group sequencing for methylation analysis. The logic of these approaches lies in methylation-specific stratification of regions of interest, in efforts to generate relevant yet feasible pools of DNA fragments, for strategized high-resolution sequence analysis.

Bisulfite sequencing following endonuclease digestion is known as Reduced Representation Bisulfite sequencing (RRBs) which selects DNA fragments of interest by size-fractionation after endonuclease digestion, this approach can select densely methylated regions, as well as gene-specific regions of interest which largely reduces generation of redundant sequence information^{81, 82}. RRBs will sequence different CpGs depending on the endonuclease(s) used. The spectrum of RRBS approaches thus are dictated by the aforementioned spectrum of endonuclease combinations which can be used to generate fragment pools of interest, dependent on the nature of the research question. The bisulfite treatment allows the subsequent employment of PCR, and so low input requirements, thus enabling the use of formalin-fixed, paraffin-embedded (FFPE) samples

Non-bisulfite-modified sequencing of endonuclease-treated or affinity-enriched DNA pools enables the mapping of relevant methylated regions, and provides some level of focus. Methyl-seq, is one of these mapping techniques which analyses the output of the HEIP assay whilst also incorporating a representational difference comparison to a pool of randomly sheared DNA fragments. Similar approaches are seen with affinity enriched DNA fragment pools, which may undergo additional array capture or padlock capture methods to filter those relevant fragments further, so as to delineate densely methylated regions of interest, within sequence-specific target regions. Padlock capture incorporates additional annealing specificity and is considered more definitive⁸³.

Whether or not bisulfite modification should precede array capture is relevant. Bisulfite modification preceding capture (BC-seq)⁸⁴ enables PCR amplification and methylation specific sequence data, however bisulfite-modified DNA fragments preclude the use of standard genomic arrays, and as such, the adapted arrays require larger spectrums of oligonucleotide probes, to increase capture efficiency of methylation state variety. Additionally, even with various probe permutations, one cannot be totally confident in capture efficiency with bisulfite-modified DNA capture.

The sporadic distribution of CpG dinucleotides across the genome makes whole-genome shotgun bisulfite sequencing cost inefficient and generates reams of redundant information. Thus, the various optimization approaches described provide a platform by which to

generate high-resolution data, but in a focused way, and as next-generation sequencing technologies continue to evolve, we will observe exponential growth in our understanding of the DNA methylome.

Gene Expression Profiling Before and After 5-Aza-2'Deoxyctidine

Gene expression profiling of cell lines treated with DNA methyltransferase inhibitors, such as 5-aza-2'deoxyctidine (5-aza-dC), is a useful and straightforward method by which to detect methylated genes^{85, 86}, based on pre and post-treatment expression changes, and is often combined with HDAC inhibition to help derepress gene expression. It was one of the first effective screens for identifying genes regulated by DNA methylation. Subsequently, the technique has been optimized to focus on genes that are differentially expressed between normal and cancer cells⁸⁷. 5-aza-dC analysis is limited to cell lines and the drug also has non-specific effects on gene expression. There are other DNA methylation inhibitors that could be also used for this purpose^{88, 89}. Furthermore, the inhibition of methylation resulting from this treatment does not result in the de-repression of all genes regulated by methylation.

Materials for Analysis of Differential Methylation Patterns

Quantitative Methylation Sensitive PCR (q-MSP)⁹⁰

1. Control DNA (normal individual)
2. Bisulfite modified control DNA (normal individual)
3. Bisulfite modified sample DNA
4. PCR buffer (Invitrogen)
5. MgCl₂ (Invitrogen)
6. 1.25 mM of each dNTP/ 50 µl reaction
7. Amplification primers complementary to modified DNA sequence
8. Amplification primers complementary to un-modified DNA sequence
9. Dual-labeled fluorescent probes (both methylated and unmethylated complimentary)
10. 5 U/µl Platinum *Taq* DNA polymerase (Invitrogen)

Gene Expression Profiling Before and after 5-Aza-2'Deoxyctidine

1. Cell lines
2. Cell media
3. 5-aza-2'deoxyctidine (Sigma)

Ultra-Deep Bisulfite Sequencing Library Preparation⁹¹

1. Isolated double-stranded genomic DNA (10 ng to 5 µg).
2. CovarisTM S2 System (Covaris, Woburn, MA, USA)
3. End polishing Enzyme 1 and 2 (SOLiDTM Fragment Library Construction Kit)
4. End-Polishing Buffer (SOLiDTM Fragment Library Construction Kit)
5. dNTP mix lacking dCTP

6. SOLiD™ Library Column Purification Kit
7. Modified double-stranded P1 Adaptor (one strand must be methylated⁹²)
8. Double-stranded P2 Adaptor (SOLiD™OLiDaptoraLibrary Oligos Kit)
9. T4 Ligase Buffer (Invitrogen, Carlsbad, CA)
10. T4 Ligase (Invitrogen, Carlsbad, CA)
11. Nick-Translation Buffer: Tris-HCl (pH 8.0), MgCl₂, NaCl
12. Exol 8.0), Fragment, 5 U/μL (Applied Biosystems)
13. 10 mM DTT
14. 0.5 M EDTA, pH 8.0
15. dNTP mix with methyl-dCTP (TriLink Biotechnologies)

Methods for Analysis of Differential Methylation Patterns

Quantitative Methylation Sensitive PCR (q-MSP)⁹⁰

1. Prepare PCR cocktail: PCR Buffer, MgCl₂, dNTPs, amplification primers and fluorescent probes, Platinum *Taq*, water
2. Add bisulfite modified sample or control to the PCR cocktail.
3. Hot-start at 95°C for 3 minutes.
4. Begin thermocycling for 40 cycles of 10 seconds at 95°C, 30 seconds at 52°C, and 30 seconds at 72°C.
5. Hold indefinitely at 4°C.

Gene Expression Profiling Before and after 5-Aza-2'Deoxyctidine

Treat cultured cells with 1 μM 5-aza-dC for 4 days, adding new media and fresh 5-aza-dC each day. PBS is used instead of 5-aza-dC for mock-treated controls. Isolated total RNA from cultured cells may be subjected to subsequent genome-wide gene expression analysis.

Notes: A 5-fold increase in a gene's level of expression is typically used to indicate that the gene is suppressed by DNA methylation in the untreated cells. Expression changes can be confirmed by RT-PCR.

Ultra-Deep Bisulfite Sequencing Library Preparation^{91–93}

Sonication

1. The Covaris™ S2 System (Covaris, Woburn, MA, USA) is recommended for fragmentation, as per manufacturer instructions to achieve an average fragment size of 165 bp (range of 150–180 bp)

(See note 9)

End-Repair

2. Combine fragmented DNA, End-Polishing Buffer, dNTP mix without dCTP, nuclease-free water, End-Polishing Enzyme 1 and 2 and incubate for 30 minutes.

⁹It is very important to keep temperature at or below 5°C while sonicating.

Purification

- 3 DNA is purified with the SOLiD™ Library Column Purification Kit

Adaptor Ligation

- 3 Combine purified and end-repaired dsDNA fragments, partially methylated P1 Adaptor, P2 Adaptor, T4 Ligase Buffer, T4 Ligase and nuclease-free water.
- 4 Incubate adaptor ligation mixture at room temperature for 15 minutes.

Purification

- 5 Purify ligated fragments with SOLiD™ Library Column Purification Kit as per manufacturer instructions.

Nick-Translation

- 6 Combine and mix the purified, ligated DNA fragments with nick-translation buffer, 10 mM DTT, dNTP mix with methyl-dCTP, Nuclease-free water, Exo-Klenow Fragment.
- 7 Incubate nick-translation for 1 hour at 16 °C
- 8 Add EDTA to stop reaction.

Purification

- 9 Purify nick-translated fragments with SOLiD™ Library Column Purification Kit as per manufacturer instructions.

(See note 10)

Miscellaneous Techniques

A handful of techniques stray from the “conventional” DNA methylation analysis template, and are briefly discussed here.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry

The application of this detection platform in methylation analyses is known commercially as the EpiTYPER assay, manufactured by Sequenom Inc⁹⁴. It involves bisulfite modification of query DNA samples, and is a comparative sequencing method which relies on base specific cleavage, and incorporates quantitative assessment at relatively high throughput, with high degrees of specificity^{95, 96}.

Immunohistochemical Staining (IHC)

Immunohistochemical staining with a monoclonal anti-5mC antibody⁹⁷, is a crude way of measuring global DNA methylation state of a cell. However, in terms of DNA methylation characterization in human cancer, it provides only limited analysis of overall global methylation patterns. The IHC assay is also dependent on antibody specificity and the antigen retrieval step has the potential to modify methylation marks.

¹⁰This prepared fragment library can then be subjected to bisulfite conversion and subsequently may be PCR amplified and transferred to a next-generation sequencing platform.

Live cell imaging of DNA Methylation Patterns

Live-cell imaging of DNA methylation is accomplished by application of a nuclear localized, methylation binding domain-containing fluorescent probe⁹⁸. This probe allows dynamic visualization of methylation patterns.

Analytical tools

Optimization strategies for methylation analyses are discussed widely in the literature, ranging from more reliable primer design programs, such as BisPrimer⁹⁹, to various adaptations which enable calculation of “true” methylation levels, such as Bayesian Tool for Methylation Analysis (Batman)¹⁰⁰, and BioCOBRA¹⁰¹, to such extensive paradigms as Comprehensive High-throughput Arrays for Relative Methylation (CHARM), which incorporates array design strategy, ideal statistical procession, and information regarding neighboring genomic regions, into the analysis. These optimization protocols are wide and varied, mirroring the vastness of currently available analytical methods. Though these fall beyond the scope of discussion in this setting, they are described very well elsewhere¹⁰².

Clinical Applications- bench to bedside

The prospect of methylation analyses reaching clinical settings is exciting in terms of potential diagnostic and prognostic capabilities, especially considering the stability of methylation patterns seen in bodily fluids, and their detectability often preceding clinical disease^{103–105}. One classic example was the finding that MGMT methylation status of gliomas could predict their response to alkylating agents¹⁰⁶. Recent clinical trials have demonstrated the effectiveness of DNA methyltransferase inhibitors for the treatment of myelodysplastic disorders¹⁰⁷, although methylation assays are not yet routinely employed to evaluate therapeutic response. Similarly, recent cancer genome studies have identified mutations in several genes in the DNA methylation pathway in myeloid leukemias, including mutations in DNMT3a and TET genes, but methylation assays are not being utilized in the clinical setting for these diseases.

Several studies have identified genome-wide DNA methylation signatures that independently predict outcome of certain cancers but these require validation in independent studies before they can be used in the clinical setting.

The realization of bench to bedside transition of DNA methylation biomarkers has been limited by some of the problems inherent to biomarker discovery research. Large well-designed clinical trials are needed to show the added value of a biomarker for diagnosing disease more effectively than standard of care. For DNA methylation assays, the lack of inter-laboratory standardizations of analytical platforms, detection sensitivity thresholds, as well as specimen handling protocols and reference samples have been obstacles. For example, quantitative MSP (qMSP) has been proposed to be one of the few ideal clinical methylation tests, due to its highly specific nature, as well as the quantitative feature which facilitates development of clinical thresholds¹⁰⁸, as well as having potential prognostic significance^{109, 110}. Despite its simplicity the reproducibility and reliability of bisulfite modification has delayed the routine use of these assays in the clinical setting. However, there are now several such assays licensed for such use (e.g. GSTpi and vimentin)^{111, 112}. The ultimate use of these assays in the clinical setting will depend on high-quality evidence of their diagnostic utility over existing diagnostic approaches.

Conclusion

In conclusion, the most commonly employed experimental approaches for DNA methylation analysis are based on bisulfite modification or methylation sensitive restriction analysis. A

variety of detection methods including sequencing and array-based methods are available. The best method to use will depend on the scientific question, cost, the type of samples to be analyzed, and depth of coverage required. As improvements continue in sequencing and array-based technologies the cost and experimental power of methylation-based methods will also improve. The clinical applications of DNA methylation detection will come as DNA methylation alterations are shown to provide important information in clinical settings.

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