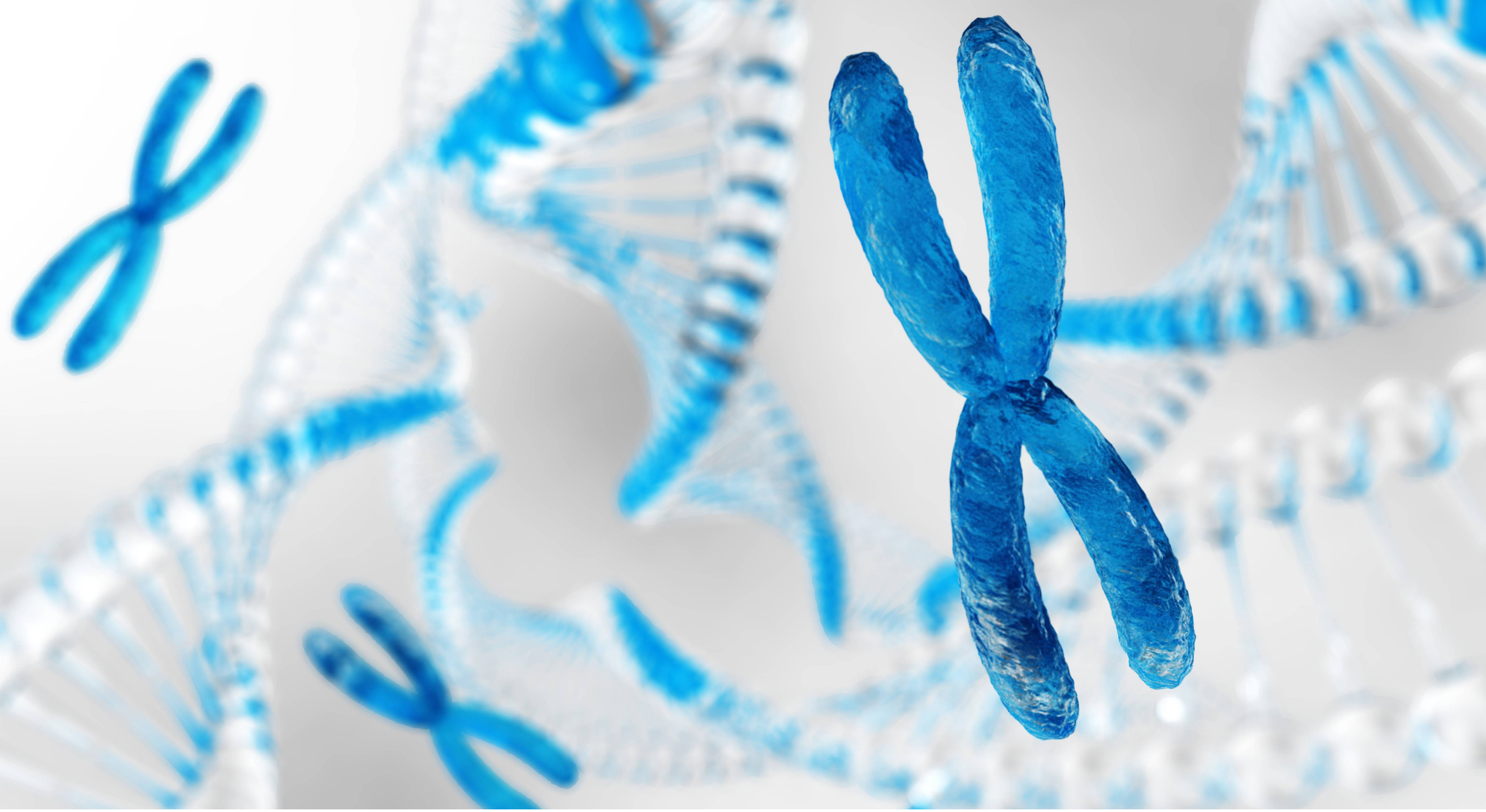

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Epigenetics
& DNA Methylation

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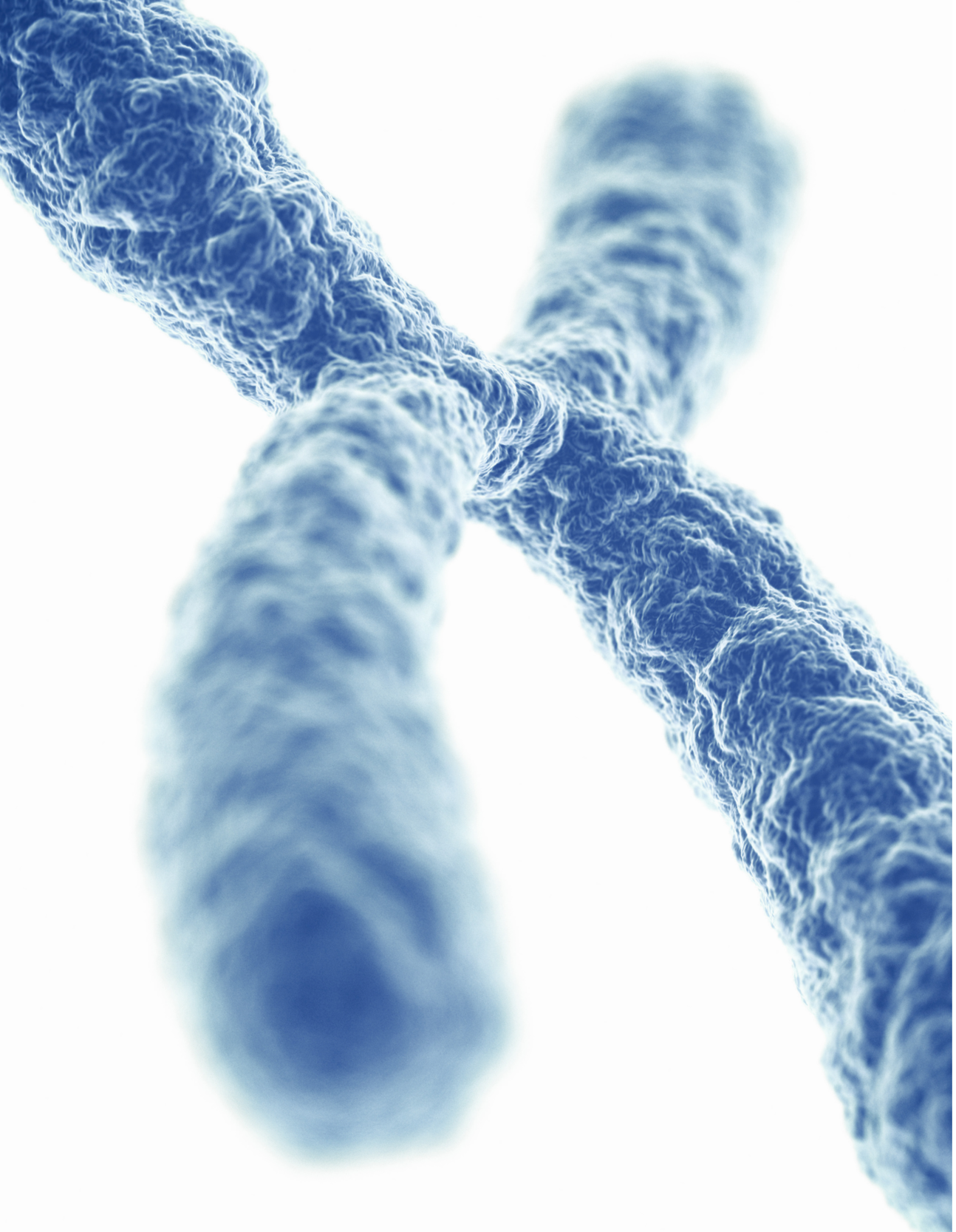
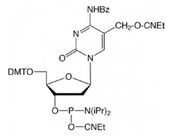


Glen Research provides a series of 5-position modified 2’-deoxycytosine monomers and a closely related 2’-deoxyU monomer for synthesis of oligonucleotides containing methyl, hydroxymethyl, formyl and carboxyl modifications useful in epigenetic and related gene expression research. This application note provides an introductory overview of epigenetic DNA and RNA modifications and an interesting recent research application using 5-Me-dC-CE in a biosensor to detect DNA methyltransferase (DNMT) activity in tumor cells.

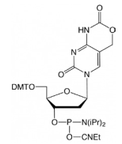
Table 1. Methyl, Hydroxymethyl, Formyl, and Carboxyl Modifications

5-Hydroxymethyl-dC-CE Phosphoramidite



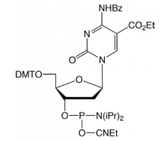
5-Hydroxymethyl-deoxycytidine (hmdC) is oxidized from 5-methyl-dC and the TET oxygenases. hmdC is found in somatic cells, germ cells, gametes, and stem cells.

5-Hydroxymethyl-dC II-CE Phosphoramidite



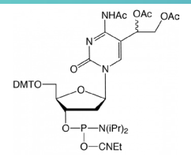
This 2nd generation molecule, 5-hydroxymethyl-deoxycytidine II CEP monomer, benefits from milder deprotection. Sodium hydroxide in MeOH/water or K2CO3 in MeOH may be used for deprotection at RT.

5-Carboxy-dC-CE Phosphoramidite



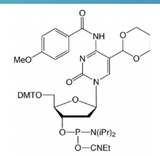
The incorporation of 5-carboxy-dC into a DNA duplex has a stabilizing effect, despite the added negative charge of the 5-carboxylic acid. In contrast, 5-carboxy-dC in the third strand of triplex forming oligos is destabilizing, lowering Tm by approximately 4-5 C°, presumably due to charge repulsion with the phosphate backbone and base-pair crowding.

5-Formyl-dC-CE Phosphoramidite



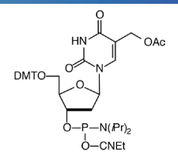
Recent studies have found that this modification may be stable within mammalian cells, suggesting a functional role other than cytosine demethylation. Deprotection with NaOH at RT improves yields and avoids formation of side-products.

5-Formyl dC III CE Phosphoramidite



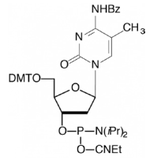
5-Formyl-dC III is a next generation monomer that features a 4-methoxybenzoyl-N protecting group. Oligos containing 5-fdC show comparable duplex melting to those containing 5-methyl-dC with the latter Tm approximately 1.3 C° higher per incorporation.

5-Hydroxymethyl-dU-CE Phosphoramidite



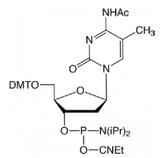
5-hydroxymethyl-dU is a marker for oxidative DNA damage that occurs by oxidative attack via peroxide radicals or ionizing radiation on the 5-methyl group of thymine. 5-hm-dU is also a deamination product of the demethylation pathway intermediate 5-hydroxymethyl-dC and may be useful in studies of epigenetic regulation involving 5-methyl-dC.

5-Me-dC-CE Phosphoramidite



One of the earliest minor bases introduced by Glen Research, 5-Me-dC may be oxidized in vivo, forming 5-hydroxymethyl-dC, 5-formyl-dC, and 5-carboxy-dC consecutively. These modified nucleosides are of immense epigenetic interest and accessible from Glen Research as phosphoramidite monomers.

Ac-5-Me-dC-CE Phosphoramidite



This 2nd generation 5-Me-dC monomer features an N4 acetyl protecting group that is fully compatible with AMA deprotection with no observed N4-Me mutation, and is also compatible with UltraMild deprotection.

Background

Epigenetics, coined by Waddington in 1942, was derived from the Greek word “epigenesis”, a term he originally used to describe the influence of multiple genetic processes on development. Waddington recognized that in developmental biology the cell phenotype, for example in a particular organ, is the product of the genotype and the epigenotype, reacting with the external environment. It was this insight, more than a decade prior to the discovery of the linear helical structure of double stranded DNA, that led him to the terminology ‘epigenetics’.

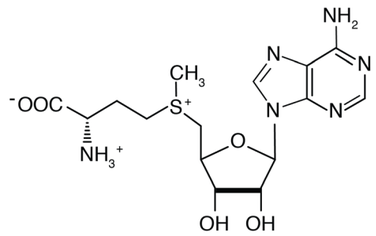


Figure 1. S-Adenosyl methionine

In these earlier times, DNA methylation and the presence of 5-methylcytosine as a minor ‘5th-base’ in DNA had long been known, while their significance was completely unknown. DNA methyltransferase enzymes are now known to transfer the methyl group from S-adenosyl methionine (SAM) to the carbon- 5 position of cytosine in DNA. It took time to realize the critical importance of this process.

It was not until 1969 that Griffith and Mahler suggested that DNA methylation may have an important role in brain memory.[2] In 1975, seminal independent publications by Riggs and by Holliday & Pugh[3] outlined similar molecular models for the switching of gene expression activities based on the enzymatic methylation of cytosine in DNA, suggesting that this DNA methylation might explain the switching on and off of genes during development.

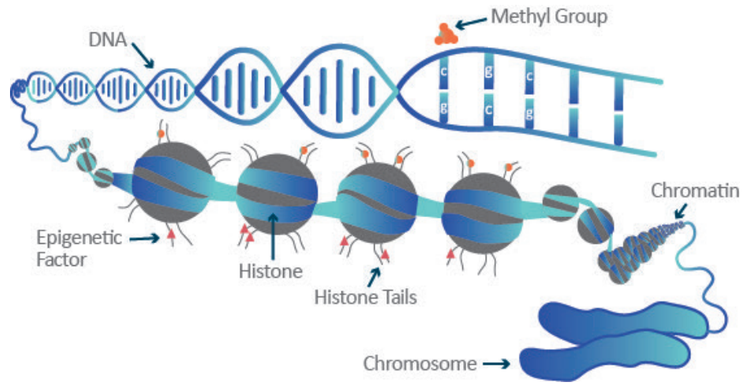


Figure 2. Chromatin structure, histones and DNA

Today, the term epigenetics refers to changes in gene expression or cellular phenotype caused by mechanisms other than changes in the genomic DNA sequence. Researchers have discovered epigenetically heritable phenotypes and multiple mechanisms controlling gene expression including DNA methylation and histone modification. In terms of gene expression, Figure 2 depicts wrapping of helical double stranded DNA around basic histone proteins, and the further folding of these structures into chromatin and then into the supercoiled chromosome.

The RNA base N6-Methyladenosine (m6A) is another modification implicated in gene expression. Discovered in 1974, m6Ahas been found in the tRNA and mRNA of an increasing number of virus, bacteria and cell types, though little was known of its sequence specific distribution and functionality. There has been a resurgence of interest recently, since the Jaffrey lab demonstrated it to be a common modification of mRNA.[4] It is now known that a series of site-specific epitranscriptomic RNA modifications are found on mRNA, and the m6A modification is understood to be dynamic and reversible, another important form of regulating gene expression through translational control.[5]

This note focuses on DNA transformations that affect the presence of 5-methylcytosine (5mC) on DNA, shown as a methyl group on a C residue at the top of the figure. The discovery of the TET oxygenase enzymes that ‘erase’ the methyl group of 5mC-marked DNA expression control elements[6] stimulated research leading to better understanding the control of gene expression through the interplay of the DNMT ‘writer’ enzymes and the TET ‘erasers’. Following a summary of this recent information, this note reviews a novel application used by researchers u to detect abnormal DNMT activity in tumor cells using an electrochemical biosensor.

TET Oxygenases

Although 5mC occurs throughout different tissues within the CpG dinucleotides of mammals, it mostly occurs in the CpG-rich promoter regions of genes — corresponding to ~4%–5% of all cytosines. The TET (ten eleven translocation) gene was initially identified from a gene translocation that can occur between chromosomes 10 and 11 creating an MLL-TET1 fusion protein in rare AML and MLL cancers. The activities of three TET enzymes on 5-methylcytosine oxidation were only recently discovered (Figure 3).

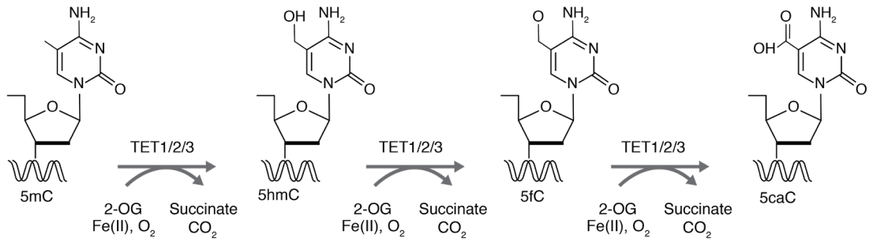


Figure 3. Stepwise oxidation of 5-methyl-cytosine

The TET oxygenases utilize ferrous iron, molecular oxygen and 2-oxo-glutarate to effect stepwise substrate oxidation. The oxidation products 5-hydroxymethyl-cytosine (5hmC), 5-formyl-cytosine (5fC) and 5-carboxy-cytosine (5caC) are also present in all tissues examined, but in low and highly variable amounts.[7] The TET oxygenase-mediated methyl-group erasing biochemistry is largely understood and it is likely that a deamination mechanism is operating, leading to thymine deglycosylase (TDG) initiation through base excision repair (BER) of the resulting mismatched Uracil.[8]

Aberrant DNA methylation is a characteristic feature of cancers and is associated with abnormal expression of oncogenes, tumor suppressor genes or repair genes. Investigations of global 5-methylcytosine content of tumors revealed hypomethylation was a common feature of carcinogenesis.[9] The activity of TET proteins is highly dysregulated in many cancers including hematologic and non-hematologic malignancies. TET1 translocations are observed in rare leukemias, and many different TET2 mutations result in loss of function in hematological disease, both in myeloid and lymphoid malignancies. Thus, the detailed interplay of protein binding events, enzyme activities and degrees of methylation remains very actively under investigation.[10]

DNA Methyltransferases

Returning the discussion to methylation, the DNA cytosine methyl group 'writers' are the DNA methyltransferases. The DNMTs involved in epigenetic control are DNMT1, DNMT3A and DNMT3B. DNMT3A and DNMT3B, the so-called de novo DNA methyltransferases, generate 5mC marks on unmethylated CpG dinucleotides (Figure 4). The 'de novo' appellation arose from recognition of their role in creating the initial methyl marks on both strands of unmethylated substrates during the early stage of embryonic development. The highly methylated CpG islands on gene-expression control-loci will have fully methylated CG sites (both strands). During replication, hemimethylated CpG sites form and are re-methylated by the maintenance methyltransferase DNMT1 (in complex with enzyme UHRF1), thus maintaining a fully methylated state in the replicated DNA.

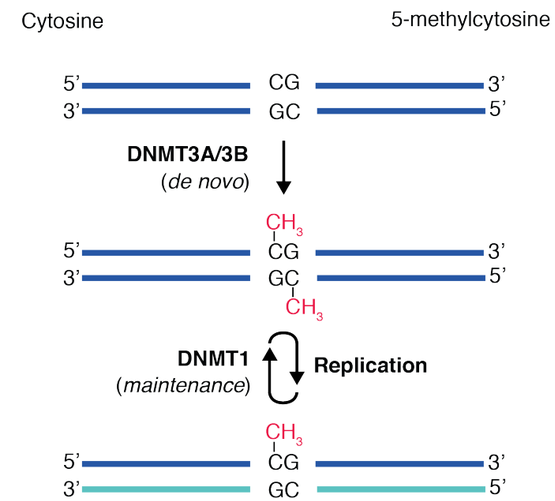


Figure 4. De novo and maintenance DNA methyltransferases

DNA Methylation and Disease

The mammalian organism's management of epigenetic alteration is crucial for retroviral element silencing, regulation of tissue-specific gene expression, genomic imprinting, and the inactivation of the X chromosome. Evidence from the past couple of decades reveals DNA methylation to be subject to diet and other factors.[11] Global hypomethylation has been suggested as a characteristic during aging, although this observation has not been reproduced by others. In addition, tracking methylation changes of a few hundred CpG sites has been shown to accurately predict chronological age. These so-called epigenetic clocks are currently the best biomarkers for predicting human mortality.[12]

Many tumor suppressor genes are observed among a subset of aberrant age-dependent hypermethylated genes. Hypermethylation can silence tumor suppressor genes and promote cancerous transformation. Aberrant hypermethylation is also observed in various chronic inflammatory conditions. For example, cigarette smoking induces hypermethylation in non-malignant esophageal tissue, while during the development of colorectal cancer (CRC), several observed DNA methylation changes affect mRNA levels and expression of genes associated with the transition from adenoma to CRC.

CRC is the 4th most common cancer and 2nd leading cause of cancer death, as 60% of patients present at stage 3 or 4, and half of these have metastatic disease. Earlier detection could have a significant impact on reducing mortality. Lynch syndrome is the most common heritable CRC and it affects individuals by predisposing them to a high risk for colorectal cancer; but the only recommended screening method for it is regular colonoscopy. Recent fecal-sample-based PCR testing has gained new proponents as a less invasive screening tool, although further benefits in user convenience and detection sensitivity would be desirable. Aberrant hypermethylation of DNA at an intragenic CpG island of the v2 region of the Septin9 gene (SEPT9) has been shown to be associated with ~97% of CRC. Based on this finding, an assay using blood plasma has been developed and FDA approved for use in CRC detection. Tumor cells shed cell-free DNA into the blood stream, and the assay detects specifically methylated cytosine residues present in these colorectal cancer tissue cells but not present in normal colon mucosa.[13]

Thus, highly sensitive methods for detecting aberrant DNA methylation are of great interest to research and diagnostic applications and may yield important new therapeutic or diagnostic human health benefits.

DNA Methyltransferase Biosensor

The Barton lab at the California Institute of Technology developed an electrochemical platform to detect the activity of the most abundant human methyltransferase, DNA(cytosine-5)-methyltransferase1 (DNMT1), and used it to detect DNMT1 activity in crude lysates from both cultured human colorectal cancer cells (HCT116) and colorectal tissue samples.[14] They chose to develop an electrochemical detection format for its relative low cost, ease of use and potential to minimize or eliminate complex or time-consuming sample processing. Existing methods of DNMT1 activity measurement either use tritiated SAM to radiolabel DNA or fluorescence or colorimetric reagents with antibodies and associated large instrumentation for detection.

A key component of their biosensor is the use of the restriction endonuclease BssHII cloned from Bacillus stearothermophilus H3, that readily cleaves both unmethylated and hemimethylated sites on double stranded DNA, but will not cleave the site containing 5-methyl-cytosine on both strands. The BssHII restriction site and cleavage positions are shown below (Figure 5) The BssHII endonuclease is an integral part of the biosensor.

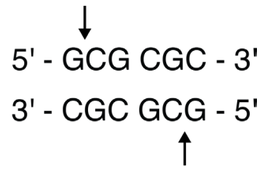


Figure 5. BssHII specificity and cleavage sites

Each electrode in the 15-electrode biosensor is prepared as a mixed monolayer with 50% azide and 50% phosphate head groups using thiol group attachment to the gold surface from a solution containing 12-azidododecane-1-thiol and 11-mercaptoundecylphosphoric acid. Synthetic hemimethyl modified duplex DNA containing a terminal alkyne group is prepared by annealing the two strands (Figure 6), and is subsequently attached to the electrode monolayer using copper click chemistry (described below). This duplex and related control duplexes, attached to the electrode surface, will be the target strand(s) for the DNMT1 enzyme present in a sample, using S-adenosyl-methionine as cosubstrate.

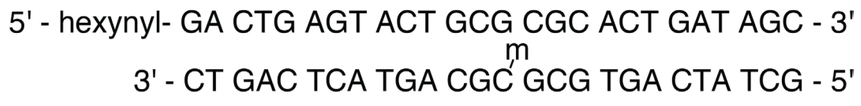


Figure 6. Hemimethyl duplex DNA target

Considerable effort went into selection of the optimal duplex DNA density on the electrode surface. At too high a density, enyzme access to some duplexes would be inadequate, and at too low a density, assay sensitivity may be reduced. Optimal low-density monolayers were found to have total DNA coverage of 20 pmol/cm^2. Optimizations also included the sequence length of the duplex, sequence length can affect signal strength, as will be apparent in the design.

In order to prepare a multiplex biosensor, duplex DNA sequences were attached to specified electrodes using electrochemically activated Cu^{+1} click chemistry. A 40 µL buffered activation solution containing $Cu(phendione)_2]^{2+}$ and duplex alkynyl-DNA is placed between the azido monolayer and patterning electrode plates and -350 mV is applied to the desired secondary electrode pins to activate the copper catalyst precursor[15] at the selected primary electrodes to catalyze azide-alkyne covalent attachment. By carrying out similar sequential attachments using differing duplex DNAs with different methylation states, the multiplex biosensor is patterned onto the individual electrodes (Figure 7). The researchers' 3x5 electrode array design "allows five experimental conditions to be run in triplicate, enabling simultaneous detection from healthy tissue and tumor tissue along with a positive control."

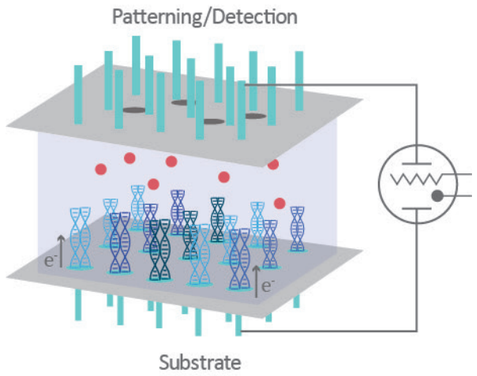


Figure 7. Patterning/Detection of biosensor

The detection equipment is a bipotentiostat with two working electrodes, a platinum wire auxiliary electrode, and a AgCl/Ag reference electrode. Measurements are performed by constant potential amperometry for 90 seconds with an applied potential of 320 mV to the patterning/detecting electrode array and −400 mV to the substrate electrode array, relative to the reference electrode.

The detection chemistry uses electrocatalytically amplified measurement of DNA charge transport (DNA CT) chemistry and has the benefit of not requiring the use of covalent labeled nucleic acid probes. In use, methylene blue (MB), a freely diffusing redox-active probe, will intercalate into duplex DNA on the electrode surface and as such is positioned for redox cycling by DNA CT. Intercalated methylene blue (MB) is reduced to leucomethylene blue (LB), the LB dissociates from the duplex and reduces ferri- to ferrocyanide, and the resulting reoxidized MB intercalates back into the DNA duplex to complete one cycle.[16] Detection at the secondary electrode of the turnover of ferricyanide, the electrocatalytic partner to methylene blue, yields measurement of the amount of DNA present on the substrate electrode. This system generally provides signal amplification of >10-fold.

Using this biosensor researchers are able to detect abnormal elevated DNMT1 activity in cultured colon carcinoma cell line lysates and also in crude tumor lysates (Figure 8). At each electrode, an initial electrocatalytic detection is made (left side of the figure) to establish the baseline current. Crude cell lysate is then added to the biosensor. When methyltransferase is present (upper blue pathway) the electrode's hemimethylated DNA restriction enzyme binding site is methylated (small green dot), now methylated on both duplex strands. In contrast, if methyltransferase is not present (lower red pathway), the hemimethylated DNA is not further methylated. Subsequent addition of BssHII to the biosensor causes cleavage of hemimethylated duplexes, however fully methylated duplexes are not cleavable by the enzyme. Thus, fully methylated duplex retains the electrocatalytic signal, as the DNA is not cleaved, allowing methylene blue intercalation and DNA-CT to occur unimpeded (blue pathway). The cleaved hemimethylated sites exhibit significant reduction in electrocatalytic signal due to loss of MB+ binding to DNA.

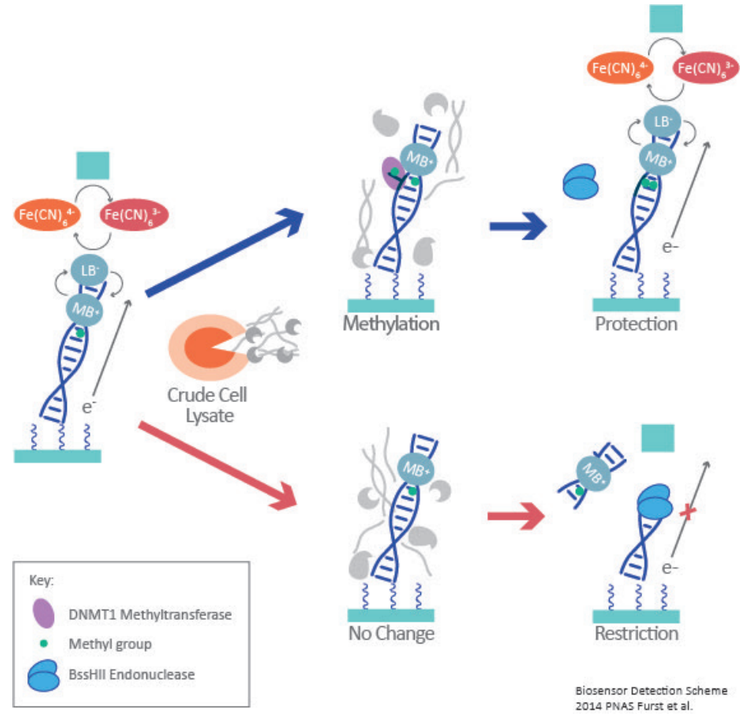


Figure 8. Detection of DNMT1 activity

In order to demonstrate specificity, substrate and cofactor requirements, various experiments were performed with the biosensor using a cultured parent (HCT116 wild-type) colorectal carcinoma cell line and a cell line that does not express DNMT1 (HCT116 DNMT1−/−). Crude cell lysates from colon cancer punch biopsies were studied, and the optimal amount of tissue for detection from these samples was determined to be ~500 µg per electrode. Typical colon punch biopsies yield 350 mg of tissue.

Typical results of colorectal carcinoma biopsy analysis are shown (Figure 9) in which carcinoma tissue and the adjacent healthy tissue were prepared, lysed, and crude lysates applied to the biosensors. The carcinoma and healthy tissue show significant differential activity. The solid colored bars represent signal remaining at hemimethylated duplex electrodes, while the hatched color bars are remaining signal at unmethylated control duplex oligo electrodes. The DNMT1 does not methylate an unmethylated duplex, and as a result, large signal is lost from unmethylated and healthy adjacent tissue, indicating significant cleavage of duplex has occurred in these two controls. The healthy adjacent lysate hemimethylated electrode (solid gray) is also cleaved, yielding low remaining signal (~30%), indicating that no significant DNMT1 activity is present in the healthy adjacent tissue sample. Meanwhile, the hemimethylated duplex electrodes that were treated with control authentic DNMT1 (green) and tumor sample lysate (blue) show complete signal protection, consistent with high DNMT1 methyltransferase activity.

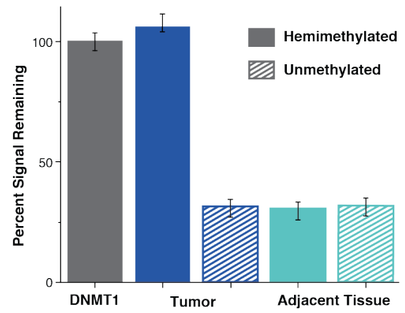


Figure 9. Tumor detection via electrochemical biosensor

These results are an elegant demonstration of the power of applying DNA-CT electrochemical detection to DNMT1, an important clinically relevant target connected to aberrant genomic methylation, and thus linked to tumorigenesis. Detection of trace proteins from crude samples is challenging due to biological sample complexity and the sensitivity needed for small clinical biopsy samples. This multiplexed electrocatalytic platform uses very small amounts of tissue and allows crude lysate to be analyzed simultaneously with controls for substrate specificity while also measuring normal and tumor tissue. The authors conclude that, "more generally, this work may be applicable to sensing other DNA modifications and certainly should represent an important step in new electrochemical biosensing technologies."

Glen Research provides 5-methylcytosine phosphoramidite for use in preparing site specific 5-methyl-dC modifications to DNA oligonucleotides. We also provide additional cytosine 5-position modification amidites that are useful for preparing oligos containing 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), 5-carboxylcytosine (5caC) and the deaminated product 5-Hydroxymethyl-dU that can be used for studies of TET oxygenases, other epigenetic research, or diagnostic testing R&D.

Table 2. Epigenetics Reagents

Item	Pack Size	Catalog No.
Ac-5-Me-dC-CE Phosphoramidite	0.25g	10-1560-02
	100µmol	10-1560-90
5-Me-dC-CE Phosphoramidite	0.25g	10-1060-02
	100µmol	10-1060-90
5-Hydroxymethyl-dC-CE Phosphoramidite	0.25g	10-1062-02
	100µmol	10-1062-90
	50µmol	10-1062-95
5-Hydroxymethyl-dC II-CE Phosphoramidite	0.25g	10-1510-02
	100µmol	10-1510-90
	50µmol	10-1510-95
5-Formyl-dC-CE Phosphoramidite	0.25g	10-1514-02
	100µmol	10-1514-90
	50µmol	10-1514-95
5-Formyl dC III CE Phosphoramidite	0.25g	10-1564-02
	100µmol	10-1564-90
	50µmol	10-1564-95
5-Carboxy-dC-CE Phosphoramidite	0.25g	10-1066-02
	100µmol	10-1066-90
	50µmol	10-1066-95
5-Hydroxymethyl-dU-CE Phosphoramidite	0.25g	10-1093-02
	100µmol	10-1093-90

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