Label-free electrochemical detection of human methyltransferase from tumors

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The role of abnormal DNA methyltransferase activity in the development and progression of cancer is an essential and rapidly growing area of research, both for improved diagnosis and treatment. However, current technologies for the assessment of methyltransferase activity, particularly from crude tumor samples, limit this work because they rely on radioactivity or fluorescence and require bulky instrumentation. Here, we report an electrochemical platform that overcomes these limitations for the label-free detection of human DNA(cytosine-5)-methyltransferase1 (DNMT1) methyltransferase activity, enabling measurements from crude cultured colorectal cancer cell lysates (HCT116) and biopsied tumor tissues. Our multiplexed detection system involving patterning and detection from a secondary electrode array combines low-density DNA monolayer patterning and electrocatalytically amplified DNA charge transport chemistry to measure selectively and sensitively DNMT1 activity within these complex and congested cellular samples. Based on differences in DNMT1 activity measured with this assay, we distinguish colorectal tumor tissue from healthy adjacent tissue, illustrating the effectiveness of this two-electrode platform for clinical applications.

DNA electrochemistry | methylation detection | electrocatalysis

DNA methylation powerfully influences gene expression in cells (1, 2). DNA methyltransferases are responsible for maintaining a genomic pattern of methyl groups, covalently added to cytosine at predominantly 5′-CG-3′ sites. Although essential for many cellular processes, aberrant methylation is associated with cancer. In particular, abnormal activity of DNA methyltransferases can lead to hypermethylation, which can silence tumor suppressor genes and promote cancerous transformations (3–6). The most abundant mammalian methyltransferase and an important diagnostic target is DNA(cytosine-5)-methyltransferase1 (DNMT1), which preferentially methylates hemimethylated DNA using the cofactor *S*-adenosyl-L-methionine (SAM) (7–10). Current measurements of DNMT1 activity require [methyl-^{3}H]-SAM to observe radioactive labeling of DNA (8, 11), or expensive fluorescence or colorimetric reagents with antibodies that require large instrumentation (12–15), both of which are significant obstacles that impede more widespread assessment of DNMT1 activity.

Traditionally, electrochemistry has been used to overcome such limitations for biomolecule detection, as electrochemical methods are low cost, portable, and require only modest instrumentation (16, 17). However, electrochemical detection schemes have typically been restricted to measurements of highly purified samples because of the increased congestion and decreased accessibility of surface (vs. solution) platforms. Electrochemistry has been used to detect nucleic acids with high sensitivity and without the need for PCR amplification in bacterial lysate and serum (18–22), but protein detection remains a challenge (23–26). In fact, although protein detection from simple serum has been accomplished (27, 28), to date no reported electrochemical systems have effectively detected active protein, of any kind, from crude cell lysate.

We have recently developed a unique electrochemical detection architecture aimed at overcoming the challenges associated with protein detection from complex biological samples. This multiplexed detection system involves a substrate plate consisting of a 15-electrode array and a complementary patterning and detection plate also containing a 15-electrode array, which combines low-density DNA monolayer patterning with the electrocatalytically amplified measurement of DNA charge transport (DNA CT) chemistry at a secondary electrode (29). The low-density DNA monolayer enables protein access to the DNA even in highly congested lysate samples, whereas electrocatalytic signal amplification markedly increases sensitivity. We use measurements of DNA CT through the DNA helices in the monolayer because of the high sensitivity of this chemistry to perturbations in base stacking caused by mismatches, lesions, and protein binding (30, 31). Methylene blue, a freely diffusing redox-active probe that is activated by DNA CT, interacts with the DNA stack and thereby reports on the integrity of DNA CT through the monolayer. We use direct detection from the patterning/detection electrode of the turnover of the electrocatalytic partner to methylene blue, ferricyanide, as a measurement of the amount of DNA present on the substrate electrode. We generally have found amplification to be >10-fold (29, 30).

Here, for the first time to our knowledge, we demonstrate the effectiveness of this platform for the detection of human DNMT1 activity from crude lysates of colorectal tumor biopsies, using a methylation-sensitive restriction enzyme to convert the methylation state of the DNA into an electrochemical signal. This strategy enables the detection of a methyl group, even though methylation itself does not significantly affect DNA CT (Fig. 1) (32, 33). Electrodes patterned with DNA containing the preferred DNMT1 methylation site (a hemimethylated 5′-CG-3′ site) are first treated with the lysate sample. Electrodes are then treated with a restriction enzyme that is sensitive to methylation at this site. If the DNA is fully methylated by active DNMT1 in the lysate sample,

Significance

Epigenetic modifications, including DNA methylation, govern gene expression. Aberrant methylation by DNA methyltransferases can lead to tumorigenesis, so that efficient detection of methyltransferase activity provides an early cancer diagnostic. Current methods, requiring fluorescence or radioactivity, are cumbersome; electrochemical platforms, in contrast, offer high portability, sensitivity, and ease of use. We have developed a label-free electrochemical platform to detect the activity of the most abundant human methyltransferase, DNA(cytosine-5)-methyltransferase1 (DNMT1), and have applied this method in detecting DNMT1 in crude lysates from both cultured human colorectal cancer cells (HCT116) and colorectal tissue samples.

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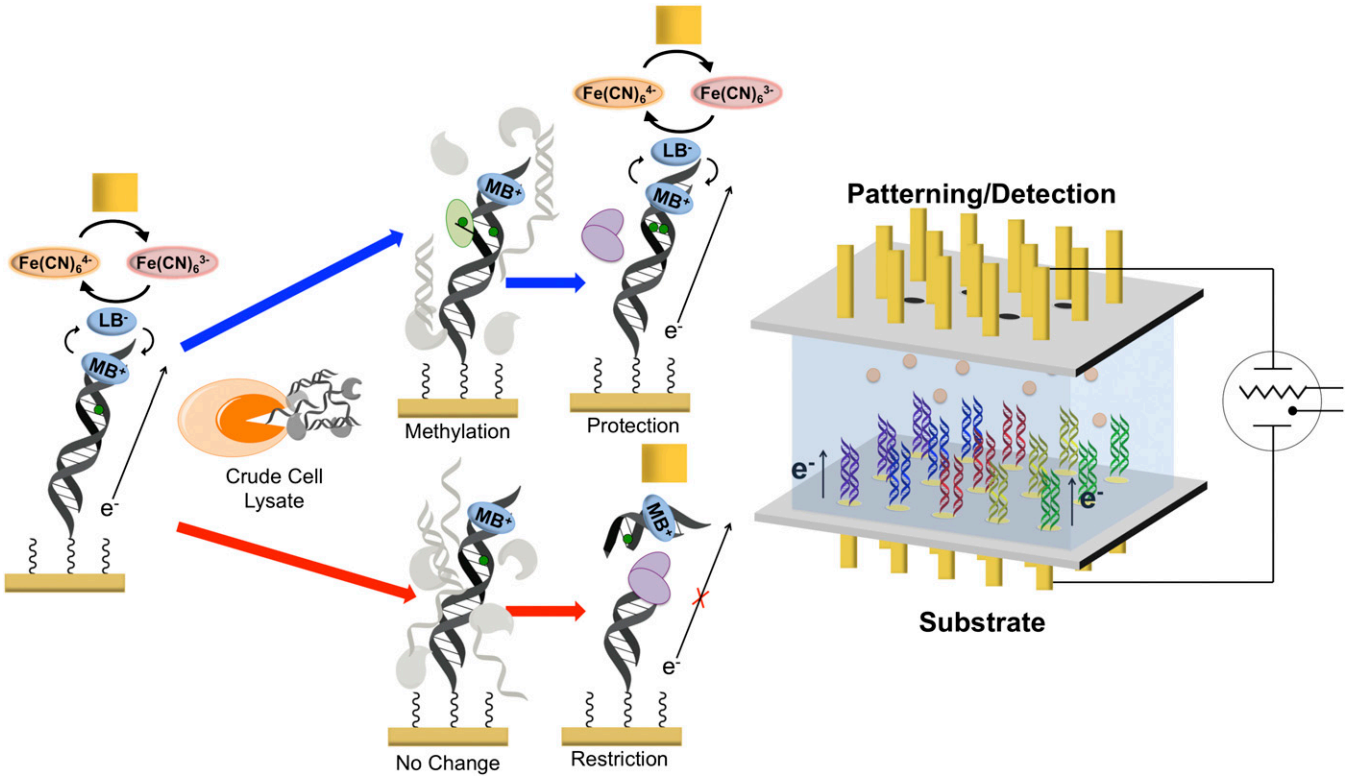

Fig. 1. Electrochemical platform (*Right*) and scheme (*Left*) for the detection of human methyltransferase activity from crude cell lysates. (*Right*) The electrochemical detection platform contains two electrode arrays, each with 15 electrodes (1-mm diameter each) in a 5 × 3 array. Multiple DNAs are patterned covalently to the substrate electrode by an electrochemically activated click reaction initiated with the patterning electrode array. Once a DNA array is established on the substrate electrode platform, electrocatalytic detection is then performed from the top patterning/detection electrode. (*Left*) Overview of electrochemical detection scheme at each electrode of the 5 × 3 array. DNA, patterned onto the bottom electrode using the copper-activated click chemistry, is electrocatalytically detected from the top electrode using methylene blue (MB+) as the electrocatalyst and ferricyanide for amplification. Crude cell lysate is then added to the surface containing the patterned DNA. If methyltransferase (green) is present (blue arrows), the hemimethylated DNA on the electrode is methylated (green dot) by the methyltransferase to a fully methylated duplex; if methyltransferase is not present (red arrows), the hemimethylated DNA is not further methylated. A methylation-specific restriction enzyme, *BssH*II (purple), is then added. If the DNA is fully methylated (blue arrows), the electrochemical signal remains protected and the DNA is not cleaved. However, if the DNA remains hemimethylated (red arrows), it is cut by the restriction enzyme, and the electrocatalytic signal associated with MB+ binding to DNA is diminished significantly.

the restriction enzyme does not cut the DNA, and there is an electrochemical signal owing to amplified DNA CT. If, in contrast, the DNA is not methylated by active DNMT1 in the lysate sample, the DNA remains hemimethylated (or unmethylated if this nonpreferred substrate is used); the restriction enzyme can then cleave the DNA, significantly decreasing the amount of DNA on the surface, and thus diminishing the electrochemical signal from DNA CT. As our electrochemical platform uses electrocatalytic signal amplification involving a freely diffusing electrocatalyst (methylene blue), in contrast with earlier work (33), the need for redox-labeled DNA is eliminated.

Using this electrochemical platform and assay, we demonstrate the efficient detection of DNMT1 activity in crude lysates from both cultured human colorectal cancer cells (HCT116) and colorectal tissue samples. Femtomoles of DNMT1 in cellular samples are rapidly detected without the use of antibodies, fluorescence, or radioactive labels. Moreover, we distinguish colorectal tumor tissue from healthy adjacent tissue through differences in DNMT1 activity, illustrating the effectiveness of this two-electrode platform for clinical applications.

Results and Discussion

Electrochemical Platform. Using a 15-pin setup, low-density DNA monolayers were formed on one set of electrode surfaces by DNA patterning from a secondary electrode. DNA substrates were optimized for length to balance the on–off signal differential with the ability of proteins to access the binding site; oligonucleotide lengths were tested with 10 nucleotides shorter and longer than the 26-mer used. For monolayer formation, thiol monolayers with 50% azide and 50% phosphate head groups first were prepared on the gold pins. We have previously characterized such low-density monolayers, and have found the total DNA coverage to be 20 pmol/cm^2 (34). Subsequently, specific DNA sequences were tethered to individual pins using electrochemically activated Cu^{1+} click chemistry (29). The secondary electrode activates the inert copper catalyst precursor only at specific locations on the primary electrode surface. Multiple sequences of DNA with different methylation states in the restriction enzyme binding site were thereby patterned onto particular electrodes (Fig. 1). The multiplexed array allows five experimental conditions to be run in triplicate, enabling simultaneous detection from healthy tissue and tumor tissue along with a positive control.

Electrochemical measurements were obtained by constant potential amperometry over 90 s. Electrodes were measured after treatment with methyltransferase, either in its purified form or as a component of crude lysate, and again after treatment with 1,500 units/mL of the restriction enzyme *BssH*II. Lysate was prepared from cultured cells through a simple treatment of cell disruption followed by buffer exchange. Purified DNMT1 was first used to establish the sensitivity and selectivity of this platform (Fig. S1) and was subsequently included alongside lysate activity measurements as a positive control. The DNA-mediated signal remains

protected from restriction, giving the same signal before and after enzyme treatment, or fully "on," when the electrode is first treated with 65 nM DNMT1 protein on a hemimethylated DNA substrate in the presence of the SAM cofactor, although protein DNMT1 is easily detectable at a 15-nM concentration with 48 ± 3% signal protection after restriction. For 65 nM DNMT1 without SAM, only 33 ± 5% signal protection is observed. Similarly, little DNA protection (31 ± 6% signal protection) is observed with the unmethylated substrate. This is explained by the strong preference of DNMT1, as a maintenance methyltransferase, for a hemimethylated substrate.

Fig. 2 shows the raw data collected for two individual electrodes treated with crude lysate, one in which the signal is on in the presence of the SAM cofactor and one in which the signal is turned "off" in the absence of cofactor, given DNA restriction in the absence of methylation. Additionally, the reproducibility of the platform is shown (Fig. 2), with the 15 individual electrodes of a single assay. Interestingly, high concentrations of lysate were found to diminish the electrochemical signal, likely due to crowding on the DNA-modified electrode, limiting access and binding of the methyltransferase. Multiple concentrations of lysate were tested (Fig. S2); a concentration corresponding to 4,000 cells per electrode was dilute enough to allow access of DNMT1 to the DNA on the surface while still containing sufficient DNMT1 to produce measurable activity.

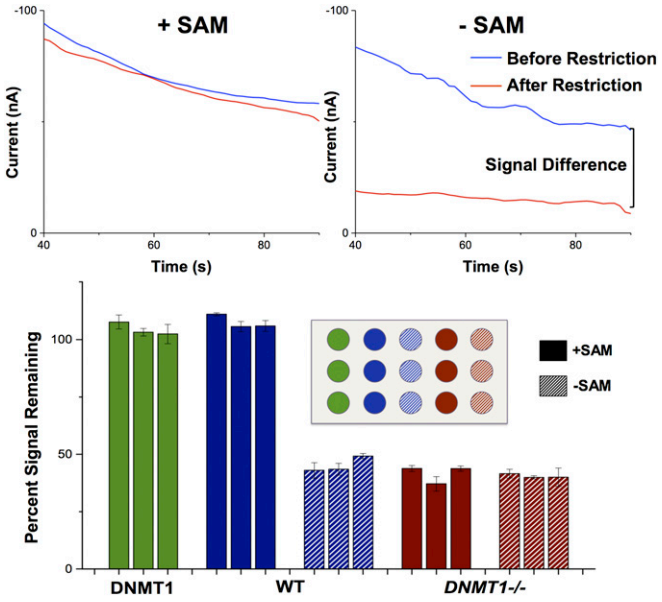

Fig. 2. Detection and reproducibility of DNMT1 activity in cell lysates using electrochemical platform. (*Upper*) Raw data from single electrodes in the presence or absence of SAM cofactor. In blue is the preliminary scan after an electrode modified with hemimethylated DNA has been treated with parent lysate, followed by treatment with 1 μM protease in phosphate buffer, both at 37 °C. In red is the scan after the electrode was treated with 1,500 units/mL *BssH*II for 1.5 h at 37 °C. (*Upper Left*) Final scan from an electrode with 160 μM SAM added to the lysate; the signals essentially overlay, indicating an "on" signal. (*Upper Right*) Final scan from an electrode without SAM added to the lysate; this produces an "off" signal. The constant potential amperometry was run for 90 s with an applied potential of 320 mV to the secondary electrode and −400 mV to the primary electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM MB and 300 μM potassium ferricyanide. (*Lower*) Reproducibility within the two-electrode multiplexed array. Orientation of the tested conditions on the 5 × 3 array is shown (*Inset*) with circular electrodes colored to correspond with activity data represented in the bar graph. Both the electrodes treated with 65 nM purified DNMT1 on hemimethylated DNA with 160 μM SAM (green) and those treated with parent (WT) lysate in the presence of SAM (solid blue) show full signal protection. On electrodes without added SAM (blue striped and red striped) and on *DNMT1* $^{-/-}$ lysate-treated electrodes in the presence of SAM (solid red), no signal protection is observed. Bar graph data are raw without standardization and error bars represent the SD over three measurements.

To further combat signal decreases caused by undesired DNA-binding proteins, after electrodes are treated with lysate, a protease treatment step is incorporated to remove remaining bound protein before the electrochemical measurements. This protease step further minimizes the possibility of remaining protein being bound either to the DNA or directly to the surface that could interfere with electrochemical measurements. Methyltransferase activity is then determined by the percent signal remaining after *BssH*II treatment. If the DNA is cut by the restriction enzyme, the signal is low, indicating little methyltransferase activity. It is noteworthy that the percent signal remaining is always nonzero because, even after restriction, a DNA fragment remains that can generate an electrochemical response with the noncovalent methylene blue redox probe; electrochemical amplification is proportional to the amount of bound methylene blue, and therefore to DNA length.

Differential Detection of DNMT1 Activity from Multiple Crude Cultured Cell Lysates. We then tested the ability of the platform to differentiate between lysate from a parent (HCT116 wild-type) colorectal carcinoma cell line and a cell line that does not express DNMT1 (HCT116 *DNMT1*$^{-/-}$). As shown in Fig. 3, specific detection of DNMT1 activity is dependent on both the methylation state of the substrate and the presence of the cofactor SAM. The "signal-on" specificity for the hemimethylated DNA substrate indicates unambiguous DNMT1 activity (maintenance methylation), and not activity by other human methyltransferases, DNMT3a or DNMT3b, which do not show this substrate preference (de novo methylation) (7). Signal is dependent on the presence of DNMT1 (purified or from parent lysate) as well as the cofactor SAM (Fig. 3*B*) and the hemimethylated substrate (Fig. 3*A*). The remaining electrodes, treated either with parent lysate without SAM, or *DNMT1*$^{-/-}$ lysate independent of the cofactor, had significantly attenuated signals after restriction enzyme treatment.

Detection of DNMT1 Activity from Human Tumor Tissue. Human biopsy tissue samples were similarly evaluated, and tumor tissue was readily distinguished from adjacent normal tissue (Fig. 3). Tissue biopsy samples were purchased from a commercial source and were thus handled and stored using conventional methods (snap freezing in liquid nitrogen after removal and storage at −80 °C for upward of 1 mo). The optimal amount of tissue for detection from these samples was found to be ∼500 μg per electrode; typical colon punch biopsies yield 350 mg of tissue (35). Samples of colorectal carcinoma tissue as well as the adjacent healthy tissue were prepared just as the cultured cell lysate, and showed differential activity with our electrochemical platform. The tumor sample, which showed greater signal protection, was sensitive both to substrate and to cofactor, consistent with high DNMT1 methyltransferase activity, similar to the cultured parent colorectal carcinoma cells. In contrast, the normal tissue sample showed low methyltransferase activity, as seen through the reduced electrochemical signal (Fig. 3). These data clearly indicate that tumors can be effectively differentiated from healthy tissue through electrochemical DNMT1 measurement with our platform. By Western blot, the relative abundance of DNMT1 in the tumor tissue compared with healthy tissue was quantitatively consistent with the electrochemical results (Fig. S3).

Lysate activities were also tested by a ^{3}H-SAM assay, and relative activities of the various samples were comparable to those determined electrochemically (Fig. S4). However, as is typical for such radioactivity assays, activity measurements observed among trials of the ^{3}H-SAM assay were extremely variable, much more so than with the electrochemical platform. Activity differences

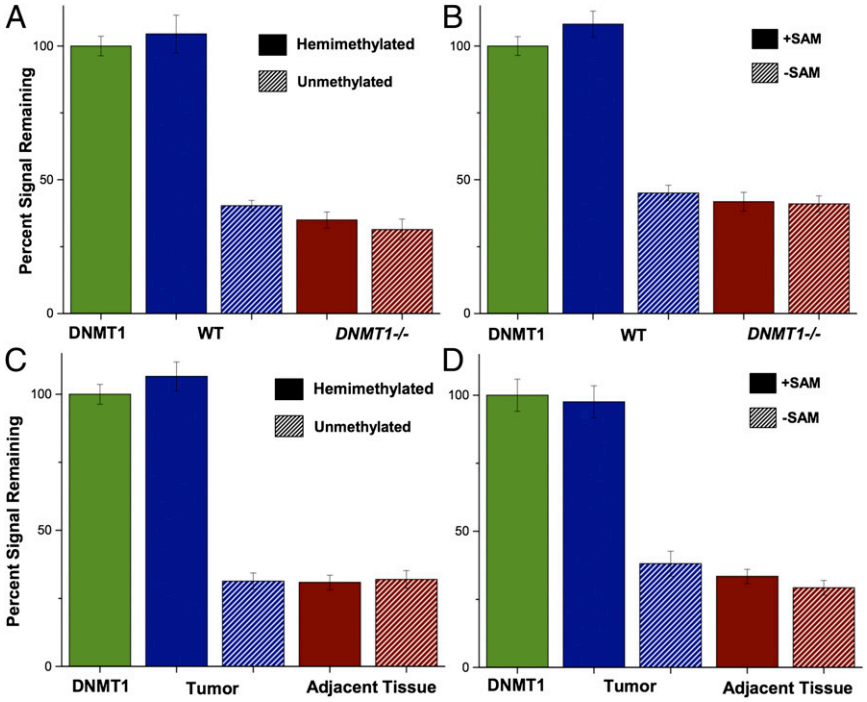

Fig. 3. Dependence of lysate activity on the DNA substrate and cofactor. The positive control is 65 nM purified DNMT1 on hemimethylated DNA with 160 μM SAM (green). All values are normalized to 100% protection of the purified DNMT1 electrodes. (*Upper*) Cultured cell lysate substrate (*A*) and cofactor (*B*) dependence. Electrodes treated with parent (WT) lysate on the hemimethylated substrate in the presence of 160 μM SAM (blue) showed full signal protection, whereas on an unmethylated substrate or in the absence of SAM (striped blue) no signal protection is observed. Independent of conditions, electrodes treated with *DNMT1* $^{-/-}$ lysate (red solid and striped) showed no signal protection. (*Lower*) Biopsy tissue substrate (*C*) and cofactor (*D*) dependence. Electrodes treated with tumor lysate on the hemimethylated substrate in the presence of 160 μM SAM (blue) showed full signal protection, whereas on an unmethylated substrate or in the absence of SAM (striped blue) no signal protection is observed. Independent of conditions, electrodes treated with normal tissue lysate (red solid and striped) showed no signal protection. The data shown represent the aggregation of three independent replicate experiments, with three electrodes per condition per experiment.

between the tumor and healthy tissue were seen only at concentrations of ∼1 mg of tissue per sample, significantly higher than what is needed for electrochemical detection. The time required to obtain the data for the ^{3}H-SAM assay was additionally substantially longer.

Implications. DNMT1 is an important clinical diagnostic target due to its connection to aberrant genomic methylation, which is linked to tumorigenesis. Direct detection of methyltransferase activity from crude tissue lysates provides an early method of cancer screening and can also inform treatment decisions. However, current approaches for detection of methyltransferase activity rely on radioactive or fluorescent labels, antibodies, and obtrusive instrumentation that limit their application in laboratories and clinics. Although electrochemical approaches generally overcome these limitations, direct detection of proteins from crude samples remains challenging because of the complexity of crude biological lysates, as well as the sensitivity required to analyze the limited material of small clinical biopsy samples.

Our electrochemical assay for DNMT1 methylation effectively circumvents these problems. Methylation is detected through the presence or absence of DNA surface restriction followed by electrocatalytic amplification. We avoid clogging the platform through the formation of low-density DNA monolayers, enabling target DNA-binding proteins in the lysate ample access to the individual DNA helices on the surface. Our platform is also sensitive and selective without the use of radioactivity, fluorescence, or antibodies through the combination of electrocatalytic signal amplification and the sensitivity of DNA CT chemistry to report changes to the integrity of the DNA. This allows for detection of DNMT1 from both cultured colorectal carcinoma cells and tissue biopsy specimens. No difficult or time-consuming purification steps are necessary, and, for each electrode, only ∼4,000 cultured cells or ∼500-μg tissue sample are required. Importantly, because of the multiplexed nature of this platform, we are able to assay for substrate specificity while simultaneously measuring normal tissue and tumor tissue lysates. Therefore, with our platform, healthy tissue is easily distinguished from tumor tissue using very small amounts of sample. More generally, this work may be applicable to sensing other DNA modifications and certainly should represent an important step in new electrochemical biosensing technologies.

Methods

DNA Monolayer Formation. The two-electrode arrays were constructed as previously reported (29). The multiplexed setup consisted of two complementary arrays containing 15 × 1-mm-diameter gold rod electrodes embedded in Teflon. Gold surfaces were polished with 0.05-μm polish before monolayer assembly. Mixed monolayers were formed on one of the plates using an ethanolic solution of 1 M 12-azidododecane-1-thiol (C_{12}thiolazide) and 1 M 11-mercaptoundecylphosphoric acid (Sigma Aldrich). Surfaces were incubated in the thiol solution for 18–24 h, followed by rinsing with ethanol and phosphate buffer (5 mM phosphate, pH 7.0). The water-soluble $[Cu(phendione)_2]^{2+}$ (phendione = 1,10-phenanthroline-5,6-dione) was synthesized by mixing two equivalents of phendione with copper sulfate in water. Covalent attachment of DNA to mixed monolayers containing 50% azide head group and 50% phosphate head group through electrochemically activated click chemistry was accomplished by applying a sufficiently negative potential to the secondary electrode. Specifically, a constant potential of −350 mV was applied to a secondary electrode for 25 min, allowing for precise attachment of the appropriate DNA to a primary electrode. Forty μL of 100 μM catalyst and 80 μL of 50 μM DNA in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) were added to the platform for covalent attachment.

Cell Culture and Lysate Preparation. HCT116 cells, either parent or *DNMT1*$^{-/-}$ (Vogelstein Lab) (9), were grown in McCoy's 5A media containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, and were grown in tissue culture flasks (Corning Costar) at 37 °C under a humidified atmosphere containing 5% CO_2.

Approximately 6 million cells were harvested from adherent cell culture by trypsinization, followed by washing with cold PBS and pelleting by centrifugation at 500*g* for 5 min. A nuclear protein extraction kit (Pierce from Thermo Scientific) was used for cell lysis, with buffer then exchanged by size exclusion spin column (10-kDa cutoff; Amicon) into DNMT1 activity buffer (50 mM Tris·HCl, 1 mM EDTA, 5% glycerol, pH 7.8). Cell lysate was immediately aliquoted and stored at −80 °C until use. A bicinchoninc assay (Pierce) was used to quantify the total amount of protein in the lysate. The total protein concentration at which the lysate was frozen was 35,000–50,000 μg/mL.

Tissue samples were obtained from CureLine. Colorectal carcinoma as well as healthy adjacent tissues were obtained. Approximately 150 mg of tissue were homogenized manually, followed by nuclear extraction, buffer exchange, storage, and quantification as described above. The total protein concentration at which the lysate was frozen was 35,000–50,000 μg/mL.

Electrochemistry. All electrochemistry was performed on a bipotentiostat (BASinc.) with two working electrodes, a platinum wire auxiliary electrode and a AgCl/Ag reference electrode. All electrochemistry was performed as constant potential amperometry for 90 s with an applied potential of 320 mV to the patterning/detecting electrode array and −400 mV to the substrate electrode array relative to a AgCl/Ag reference electrode with a platinum auxiliary electrode. All scans were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide. Scans were taken of each of the 15 secondary pin electrodes, and the reported variation in the data represents the SE across 3 measurements of 3 electrodes, all at a given condition.

To incubate electrodes with desired proteins, a 1.5-mm-deep Teflon spacer was clipped to the primary electrode surface. Each electrode is isolated in an individual well that holds 4 μL of solution. For methyltransferase activity detection, three electrodes on the device were always incubated with 65 nM DNMT1 with 160 μM SAM and 100 μg/mL BSA as a positive control. For electrodes incubated with lysate, lysate was either directly combined with SAM to a final SAM concentration of 160 μM or the lysate was diluted in DNMT1 activity buffer to the desired total protein concentration and then combined with SAM to a final SAM concentration of 160 μM. For the tissue lysate, 50 μg/mL BSA was also added. Each electrode had the desired solution added to the well and incubated at 37 °C for 1.5 h in a humidified container. The substrate electrode array was then treated with 1 μM protease solution in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) for 1 h. The surface was then thoroughly rinsed with phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) and scanned. The electrodes were subsequently incubated with the restriction enzyme *BssH*II at a concentration of 1,500 units/mL for 1.5 h at 37 °C in a humidified container. *BssH*II was exchanged into DNMT1 activity buffer by size exclusion column (10 kDa, Amicon). The electrodes were again rinsed with phosphate buffer and scanned.

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