SUPPORTING INFORMATION

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Fluorescence Probes of ALKBH2 Measure DNA Alkylation Repair and Drug Resistance Responses

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Abstract: The DNA repair enzyme ALKBH2 is implicated in both tumorigenesis as well as resistance to chemotherapy in certain cancers. It is currently under study as a potential diagnostic marker and has been proposed as a therapeutic target. To date, however, there exist no direct methods for measuring the repair activity of ALKBH2 *in vitro* or in biological samples. Here we report a highly specific, fluorogenic probe design based on an oligonucleotide scaffold that reports directly on ALKBH2 activity both *in vitro* and in cell lysates. Importantly, the probe allows us to monitor cellular regulation of ALKBH2 activity in response to treatment with the chemotherapy drug temozolomide through a simple fluorescence assay, which has only previously been observed through indirect means such as qPCR and Western blots. Furthermore, the probe provides a viable high throughput assay for drug discovery.

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1. Materials and Methods

Instrumentation: Semi-preparative HPLC was performed on an LC-20AD Shimadzu liquid chromatography system, equipped with a SPD-M20A diode array detector and a CBM-20A system controller and using a SunFire reverse phase C18 column (Waters). Fluorescence emission and excitation spectra were recorded on a Jobin Yvon-Spex Fluorolog 3 spectrometer with an external temperature controller. Experiments carried out on microplates were recorded by a Fluoroskan Ascent Microplate Fluorometer (ThermoFisher Scientific). DNA synthesis was carried out on an Applied Biosystems 394 DNA/RNA synthesizer. Oligonucleotide masses were determined by MALDI-TOF at the Stanford University Protein and Nucleic Acid Facility on a Voyager DE RP instrument (Applied Biosystems). Oligonucleotide concentrations were determined by UV-absorption on a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). Melting curves were recorded on a Varian Cary100 UV/Vis spectrophotometer equipped with a thermoprogrammer.

*Chemicals***.** All chemicals were purchased from Combi-Blocks, Sigma Aldrich, Ark Pharm or Oakwood Chemical and used without further purification. All phosphoramidites (except $_{\alpha}$Y, $_{\beta}$Y and ^{m1}A), CPG supports and DNA synthesis reagents were purchased from Glen Research. All cell culture reagents except FBS were purchased from Gibco (ThermoFisher Scientific). FBS was purchased from Corning.

Probe Synthesis and Purification: All probes were synthesized at a 1 µmole scale on 3'-phosphate CPR II CPG columns using standard β-cyanoethyl phosphoramidite chemistry. Probes were synthesized the 3'-5' direction using the DMT-off method. Coupling times were 300 s for natural bases (dA, dT, dC and dG) and 999 s for all other phosphoramidites. Modified β-cyanoethyl phosphoramidites $_{\alpha}$Y and $_{\beta}$Y were synthesized as previously reported.[1] Alpha and beta anomers were separated by FCC following acid catalyzed epimerization[2] and anomeric purity was confirmed by NMR. The modified β-cyanoethyl phosphoramidite ^{m1}A was synthesized as previously reported.[3] Following DNA synthesis, CPGs were treated with 50% triethylamine/acetonitrile for 4 hours to remove β-cyanoethyl groups. CPGs were then rinsed 3x with acetonitrile (1 mL) and dried under argon. To avoid base catalyzed dimroth rearrangement of ^{m1}A to ^{m6}A, Oligonucleotides were cleaved and base deprotected with 0.05 M K_2CO_3 in dry

methanol (Glen Research) at room temperature for 48 hours. The deprotection mix was neutralized with glacial acetic acid (6 µL per mL) and dried by SpeedVac. All probes were purified by semi-preparative HPLC on a reverse phase C18 column (5 µM, 10 mm x 250 mm) using a TEAA (0.1 M, pH 7.4) acetonitrile solvent system. The following gradient was used to purify all probes: 5-95% acetonitrile at 2.0 mL/min flow over 40 minutes. The collected peaks were concentrated by SpeedVac and identified by MALDI-TOF (Table S3). Probe concentrations were determined by the extinction coefficient of pyrene at 345 nm (43,000 $M^{-1}cm^{-1}$ for single pyrene and 86,000 $M^{-1}cm^{-1}$ for pyrene excimer).

ALKBH2 Protein Expression and Purification: ALKBH2 was purified using the overexpressing pET28a-ALKBH3 plasmid in Rosetta (DE3) cells (Novagen) with selection using chloramphenicol (34 µg/mL) and kanamycin (25 µg/mL). From a single colony, 20 mL of a 50 mL overnight culture was used to innoculate 500 mL of Luria-Bertrani medium in a 2 L Erlenmeyer flask containing the appropriate antibiotics for selection. The cells were grown at 37 °C to an OD_{600nm} of 0.6. ALKBH2 protein production was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM and continued for 5 hours with vigorous shaking for aeration. Cells were harvested by centrifugation and washed with phosphate buffered saline (150 mM NaCl, 10 mM phosphate, pH 7.4) and stored at -20 °C until use. Cells were lysed in 10 volumes of lysis buffer in mL (100 mM NaCl, 50 mM phosphate pH 8, 5 mM β-mercaptoethanol, and 0.5 % TritonX 100) as compared to the wet cell pellet weight in grams. Egg white lysozyme (Sigma-Aldrich) was added to 0.2 mg/mL final concentration and the cells were incubated on ice for 20 minutes and then at 37 °C for 20 minutes. Following the incubation at 37 °C, DNase I (Sigma-Aldrich, molecular biology grade) was added to a final concentration of 1 µg/mL and incubated on ice for 10 minutes and then at 37 °C for 10 minutes. The lysate was sonicated briefly in a Bioruptor Sonicator and then centrifuged at 16,000 *g* for 20 minutes at 4 °C in microfuge tubes. The supernatants from the individual tubes were combined into a 15 mL centrifuge tube and incubated with 1 mL of DEAE Sepharose Fast Flow resin (GE Healthcare) prepared according to the manufacturer. The lysate and resin were incubated on a rotational shaker at ~10 °C for 20 minutes. The suspension was centrifuged at ~3000 g for 5 minutes at 4 °C to pellet the resin. The resin was washed twice with 10 mL of the lysis buffer and all 3 fractions were combined. The concentration of the combined fractions was adjusted to 20 mM imidazole and

loaded onto a 1 mL Ni-NTA Hi-Trap column (GE Healthsciences). The column was washed with the lysis buffer with 20 mM imidazole. A gradient was performed between 100-200 mM imidazole and between 200-500 mM imidazole (15 ml total volume) by collecting 0.5 mL fractions in 1.5 mL polypropylene centrifuge tubes. Fractions containing ALKBH2 were identified by electrophoresis and pooled. The pooled ALKBH2 fractions were dialyzed twice against 20 volumes of storage buffer (150 mM NaCl, 50 mM NaH_2PO4 pH 7, 5 mM β-mercaptoethanol, 50% glycerol) and stored at -20 °C. The concentration of protein was determined using a BioRad Protein Assay reagent using bovine serum albumin as a standard.

General probe reaction conditions with purified enzyme: All probe reactions were performed in 50 mM HEPES KOH buffer (pH 8.0) at 37 °C. Unless otherwise noted, all reactions were supplemented with 75 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM 2-oxoglutarate, 2 mM sodium ascorbate, 50 μg/mL BSA and 0.4 mg/mL Catalase (Bovine liver, Sigma Aldrich). Unless otherwise noted, all reactions with hairpin probes **12**, **13** and **13p** were also supplemented with 1.5 mM $MgCl_2$. Prior to being used, all hairpin probes were diluted to a concentration of 10 μM in 50 mM HEPES KOH (pH 8.0) buffer with 1.5 mM magnesium and annealed at 95 °C for 3 minutes and cooled by setting the heat block on the benchtop at room temperature. Unless otherwise noted, all reactions with purified enzyme were carried out using 500 nM enzyme and 1 μM probe. All fold change values were determined by comparing the fluorescence emission spectra at 345 nm excitation after a 5-minute preincubation period to the emissions spectra following repair. Single pyrene probes were monitored at emission of 385 nm and pyrene excimer probes at 480 nm. Microplate reader experiments were carried out with excitation at 355 nm and emission monitored at 460 nm.

Cell Growth and Lysate Preparation: All cells were grown in DMEM supplemented with FBS (10%), penicillin (100 U/mL), streptomycin (100 U/mL) and L-Glutamine (4 mM) in a humidified incubator at 37 °C with 5% CO_2. For experiments with temozolomide (TMZ) (Combi-Blocks), cells were grown to ~70% confluence and then grown for 24 more hours with fresh media supplemented either with or without 100 μM TMZ (<0.1% DMSO). To prepare lysates, cells were collected in PBS by scraping and the protocol for the CellLytic™ NuCLEAR Extraction Kit (Sigma Aldrich) was used with Roche complete mini EDTA-free protease inhibitor tablets. Briefly, cells were grown to 70-80% confluency and harvested by scraping (~$5x10^7$). Cells were rinsed

twice with cold PBS and swelled in hypotonic lysis buffer for 15 minutes on ice (10 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 M DTT and 1x protease inhibitor). Cells were lysed by repeated passage through a 25-gauge needle and the cytosolic fraction collected. Nuclear proteins were extracted from the nuclear pellet by shaking with a high salt nuclear extraction buffer for 30 minutes (20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 M DTT, 25% glycerol and 1x protease inhibitor). The nuclear and cytosolic fractions were then combined and total protein was determined by Bradford assay.

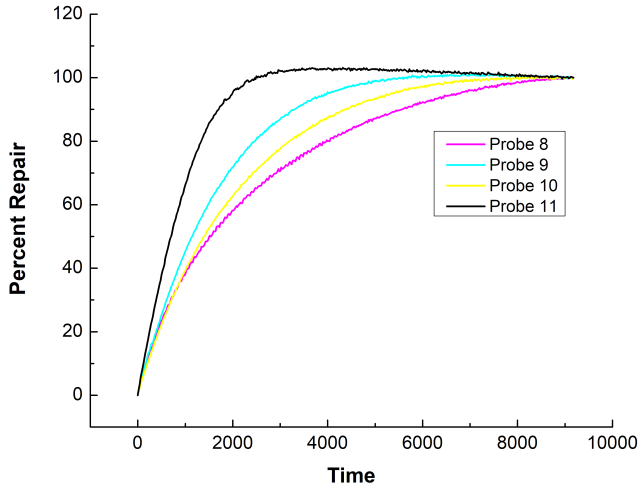
Fluorescence measurements with cell lysates: Cell lysate experiments were conducted at 37 °C on a 384-well plate at a volume of 60 µL using 1 mg/mL crude protein and 2 µM probe. Experiments were conducted in the hypotonic lysis buffer supplemented with 75 µM $Fe(NH_4)_2(SO_4)_2$, 1 mM 2-oxoglutarate, 2 mM sodium ascorbate, 50 µg/mL BSA and 0.4 mg/mL Catalase. Fluorescence intensity was monitored over 24 hours with 355 nm excitation and 460 nm emission. A transparent, adhesive plate cover was used to prevent evaporative loss during the course of the experiment.

Pyridine 2,4-dicarboxylic acid (PDCA) IC_{50} measurement: General probe reaction conditions were used on a 384 well plate at a 60 µL reaction volume. Varying concentrations of PDCA were added (0.1-200 µM) to the reaction mixture (DMSO <1%) and the initial velocity was measured over the first 2 minutes of the reaction and compared against the control rate. The resulting dose-response curve was fitted to the Hill Equation (OriginPro 8.5) to calculate IC_{50}.

Oligo Duplex IC_{50} measurement. Random oligonucleotide sequences were generated and synthesized along with their compliments (Integrated DNA Technologies). Duplexes were formed by annealing equimolar quantities of each strand in 50 mM HEPES buffer (pH 8.0) with 1.5 mM $MgCl_2$ (95-25 °C with a ramp of 1 °C/min). Varying concentrations of oligonucleotide duplex were added (5-200 nM) and the initial velocity was measured over the first 2 minutes of the reaction and compared against the control rate. The resulting dose-response curve was fitted to the Hill Equation (OriginPro 8.5) to calculate IC_{50}.

Michaelis Menten Analysis: The Michaelis Menten curve for probe **13p** was generated using the general probe reaction conditions on a plate reader at a 60 µL volume. Each reaction used 50 nm enzyme and varying amounts of probe. The initial velocity of each probe concentration was

calculated as fluorescence intensity fold change per second over the first 2 minutes of the reaction. The resulting curve was fit to the Hill Equation (OriginPro 8.5) to calculate the K_m value.



	Probe 8	Probe 9	Probe 10	Probe 11
ALKBH2 $t_{1/2}$ (sec)	N.C.	165	200	200
ALKBH3 $t_{1/2}$ (sec)	1500	1140	1400	700

Figure S1. Time course of ALKBH3 with probes **8-11** demonstrates that the single stranded polyA probes are suitable substrates for ALKBH3 regardless of length. ALKBH3 appears to prefer the longest probe while the shortest probe is repaired slowest (not repaired at all by ALKBH2).

Probe	1.5 mM Mg^{2+}	Enzyme	Init. Rate	Selectivity
Probe **13**	+	ALKBH2	1.12E-01	115.61
		ALKBH3	9.66E-04	
	-	ALKBH2	9.80E-02	27.76
		ALKBH3	3.53E-03	
Probe **13s**	+	ALKBH2	4.47E-02	2.26
		ALKBH3	1.97E-02	
	-	ALKBH2	6.78E-02	2.64
		ALKBH3	2.57E-02	

Probe	Probe Sequence
13	CGC TTA SSA TTT GCG Hex CGC AAA $^{m1}A_{\beta}Y_{\beta}Y$ AAA GCG
13s	CGC AAA $^{m1}A_{\beta}Y_{\beta}Y$ AAA GCG

Table S1. Probe **13** and its single-stranded analogue **13s** were tested for their selectivity between ALKBH2 and ALKBH3 in the presence and absence of 1.5 mM $MgCl_2$. The initial velocity was measured as the slope of the fluorescence time course over the initial 2 minutes of the reaction. Selectivity is calculated based on the initial velocity of ALKBH2 over ALKBH3. While the addition of 1.5 mM $MgCl_2$ to the reaction buffer does not affect the selectivity of probe **13s** (the initial velocities of both enzymes are reduced equally), the selectivity of probe **13** is increased 4-fold over the selectivity of the probe in buffer without the divalent cation. The increased selectivity arises both an increase in the initial velocity ALKBH2 and a significant decrease in the rate of ALKBH3 repair.

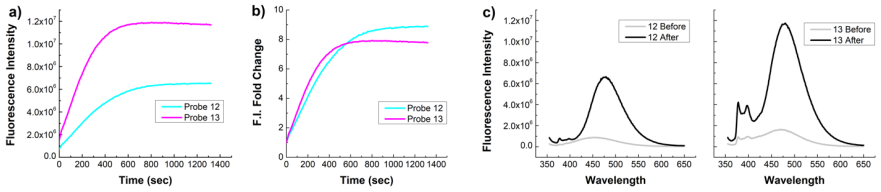


Figure S2. Comparisons of probes **12** and **13** demonstrate that the replacement of neighboring thymidine residues on the complimentary strand reduce quenching but have a minimal effect on fold change in response. (a) Fluorescence time course of probes **12** and **13** with ALKBH2. (b) Fold increase in fluorescence intensity of probes **12** and **13** with ALKBH2. (c) Fluorescence spectra of probes **12** and **13** before and after repair by ALKBH2. Pyrene monomer emission between 360-400 nm is particularly strongly quenched by the neighboring thymidine as seen in probe **12**.

Duplex	Sequence	Duplex IC_{50}
D1	5′ GCG GAC GGT GGG CAA 3′ 3′ TTG CCC ACC GTC CGC 5′	15.24 nM
D2	5′ CCG ATG ACG TCA GCC T 3′ 3′ AGG CTG ACG TCA TCG G 5′	16.18 nM
D3	5′ GAA TGA CTG ACT AAC TGA 3′ 3′ TCA GTT AGT CAG TCA TTC 5′	<20 nM

Table S2. Sequences and apparent IC_{50} values of DNA duplexes used to study inhibition of ALKBH2 repair of probe **9**.

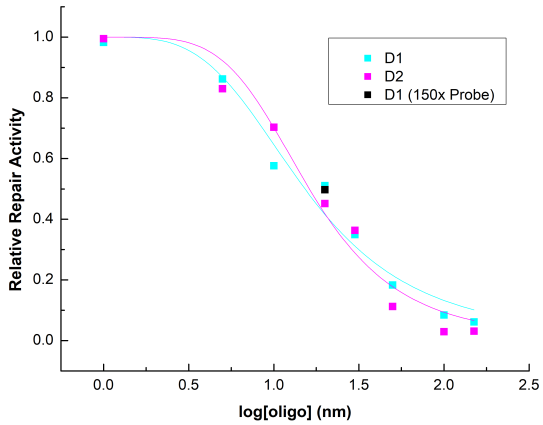


Figure S3. Dose response curves of ALKBH2 repair activity on probe **9** with varying amounts of duplex DNA added. Even in the presence of 150-fold excess probe, the D1 duplex still caused the same reduction in repair activity of probe **9**. A full dose response curve was not generated for duplex D3; however, results from single data points indicate its IC_{50} value to be below 20 nM.

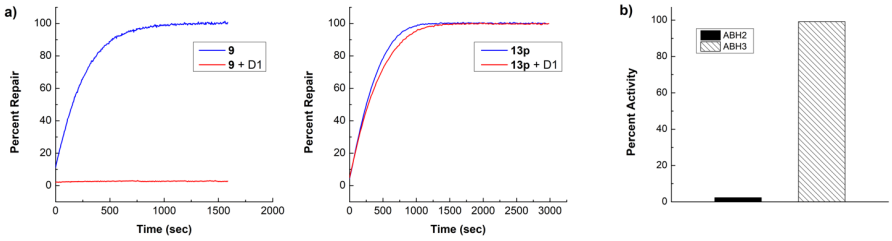


Figure S4. a) Repair activity of ALKBH2 on probes **9** and **13p** in the presence of doubple-strnaded DNA D1 (0.5 µM; no magnesium). b) Relative percent activity of ALKBH2 and ALKBH3 on probe **9** in the presence of 0.5 µM D1 (no magnesium) as measured by initial velocity. ALKBH3 is unaffected by D1 while ALKBH2 has essentially no activity.

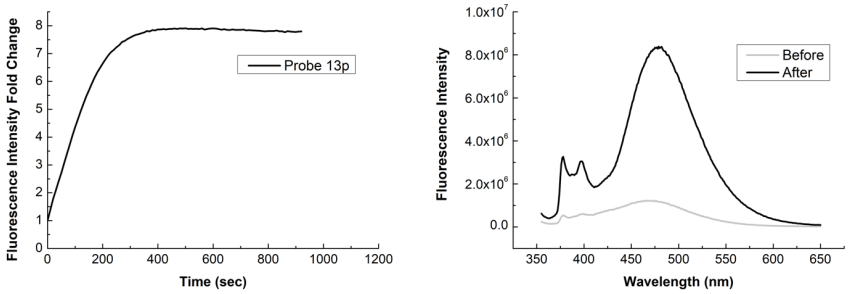


Figure S5. Fluorescence time course of probe **13p** with ALKBH2 (+1.5 mM $MgCl_2$) and fluorescence emission spectra before and after incubation with ALKBH2 (excitation 345 nm).

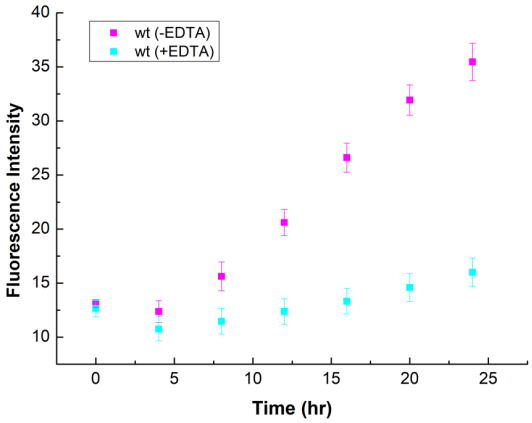


Figure S6. Fluorescence response of wt MEF cell lysates with probe **13p** with and without 1 mM EDTA added to the buffer.

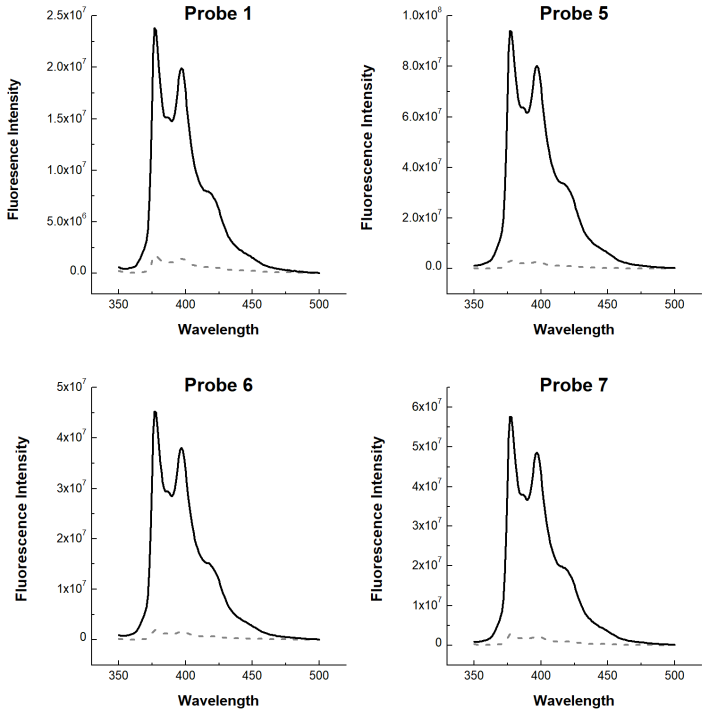


Figure S7. Emission spectra of single pyrene probes **1,5,6,7** (excitation 345 nm) before (dashed lines) and after (solid lines) reaction with ALKBH2.

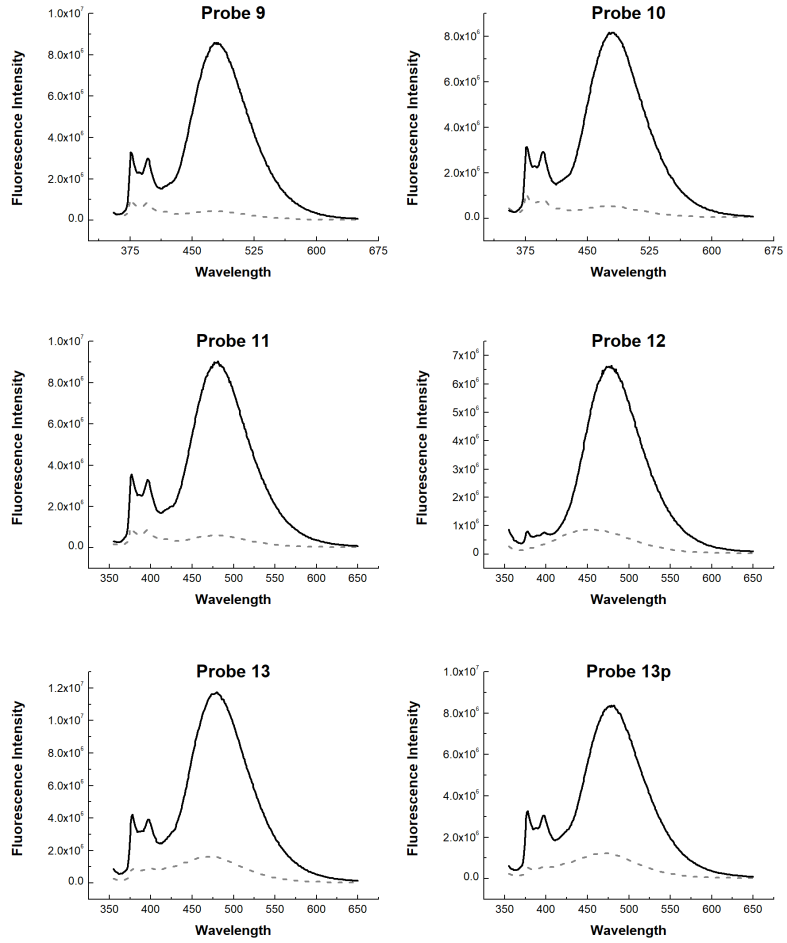


Figure S8. Emission spectra of pyrene excimer probes **9-13p** (excitation 345 nm) before (dashed lines) and after (solid lines) reaction with ALKBH2.

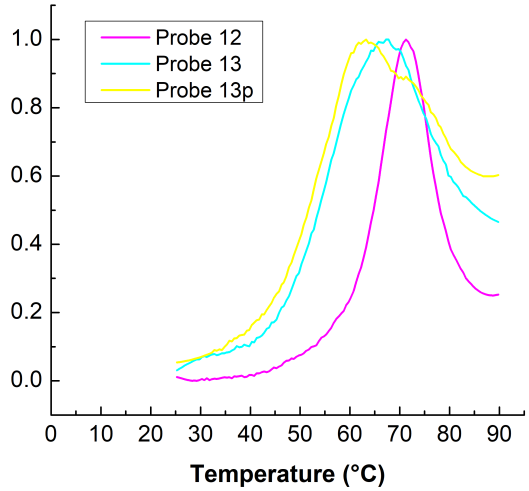


Figure S9. Normalized first derivatives of melting curves for duplexes **12**, **13** and **13p**. Probes were first annealed in buffer (50 mM HEPES, 1.5 mM $MgCl_2$) by heating to 95 °C for 3 minutes and cooling to 25 °C at a rate of 1 °C/minute. Melting curves were then collected by monitoring at 260 nm and ramping from 25 °C to 90 °C at a rate of 0.5 °C/minute. Comparing probes **12** and **13** demonstrates that replacing the neighboring thymidines around pyrene with deoxyadenosine mispairs caused a ~4 degree lowering of T_m as well as a broadening of the melting transition. Adding 2'-O-methyl protection to probe **13p** lowered T_m further by ~4 degrees.

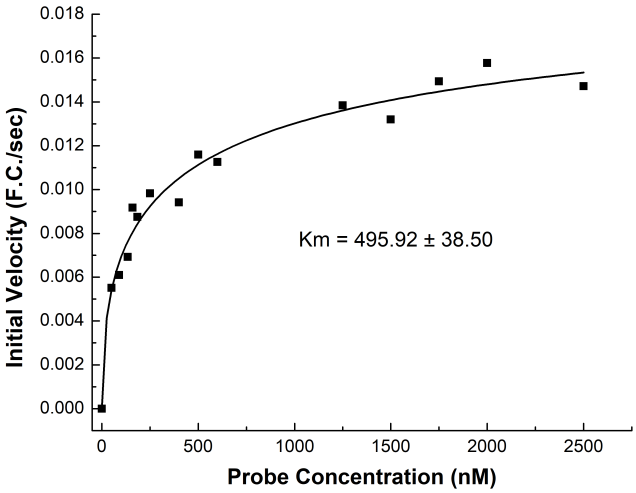


Figure S10. Probe **13p** K_m measurement with ALKBH2.

Table S3 List of probe masses observed by MALDI-TOF

Probe Name	calc'd mass	found	Species
MAQ	3299	3298	$[M]^+$
1	3232	3232	$[M]^+$
2	3231	3233	$[M]^+$
3	3232	3233	$[M]^+$
4	3857	3856	$[M]^+$
5	4798	4799	$[M]^+$
6	6364	6365	$[M]^+$
7	7930	7934	$[M]^+$
8	3299	3300	$[M]^+$
9	4865	4864	$[M]^+$
10	6432	6429	$[M]^+$
11	7999	8000	$[M]^+$
12	9530	9536	$[M]^+$
13	9548	9550	$[M]^+$
13p	9669	9716	$[M +2Na]^+$

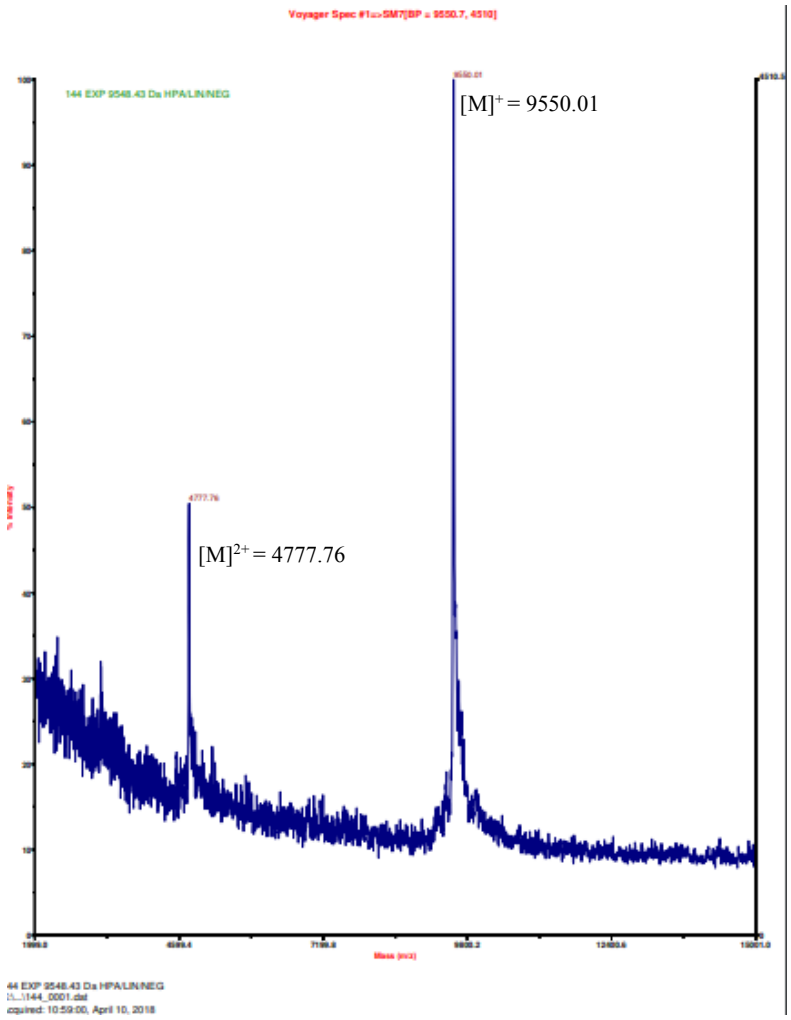


Figure S11. Representative MALDI-TOF spectrum for probe **13**.

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