Systematic evaluation of split-fluorescent proteins for the direct detection of native and methylated DNA

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Materials and methods

General materials

All materials were obtained from Sigma-Aldrich unless otherwise noted. Empty expression vectors, pETDuet and pRSFDuet, and the *Escherichia coli* BL21(DE3) cell line were purchased from Novagen. 2XYT media was purchased from Becton Dickinson, and zinc chloride ($ZnCl_2$) was obtained from EM Sciences. Ampicillin, dithiothreitol (DTT), isopropyl-β-D-thiogalactopyranoside (IPTG), Urea, and Tris-Base Ultrapure, were obtained from Research Products International. Plasmids containing full-length Cerulean (plasmid 11930)[1] and mCherry (plasmid 13095)[2] were purchased from Addgene.

[1] Cole, N. B.; Smith, C. L.; Sciaky, N.; Terasaki, M.; Edidin, M.; Lippincott-Schwartz, J. *Science (Wash.)* **1996,** *273*, 797.
[2] Hamacher-Brady, A.; Brady, N. R.; Logue, S. E.; Sayen, M. R.; Jinno, M.; Kirshenbaum, L. A.; Gottleib, R. A.; Gustafsson, A. B. *Cell Death Differ.* **2007,** *14*, 146.

Cloning and protein expression

All SEER proteins were cloned into the pETDuet dual expression vector, with PBSII, PE8B, or MBD2 fused to the C-terminal FP fragment in the first multiple cloning site as a hexa-histidine fusion and Zif268 fused to the N-terminal FP fusion in the second multiple cloning site. Additionally the FP-Zif268 gene was cloned into pRSFDuet as a hexa-histidine fusion for each of the fluorescent proteins investigated, excluding NGFPuv-Zif268, to facilitate purification. Protein expression and purification were performed as previously described (ref 13). Briefly, in all cases the plasmid containing the FP fusion(s) was transformed into *E. coli* BL21(DE3) cells. 16-hour cell cultures were back diluted to an A_{600} of 0.05 into 2XYT media supplemented with the appropriate antibiotic (ampicillin or kanamycin) and 100 μM $ZnCl_2$. Cultures were induced at an A_{600} between 0.5 and 1.0 with 1 mM IPTG. Proteins were isolated from inclusion bodies via resuspension in Buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 μM $ZnCl_2$, 1 mM DTT) supplemented with either 4 or 8 M urea, depending on protein solubility. Proteins were purified under denaturing conditions on a 1 mL NiNTA (Qiagen) column. Concentrations were determined using A_{280} measurements corrected for nucleic acid content, and purity was assessed with SDS-PAGE analysis.

dsDNA production

Complementary target oligonucleotides were ordered HPLC-purified from Integrated DNA Technologies. The DNA was resuspended in H_2O and annealed to a final concentration of 50 μM in 1x BamHI buffer (New England Biolabs: 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$). Oligonucleotides were annealed in a Techne Genius thermocycler using the following protocol: heating to 95°C for 7 min, cooling to 56°C at a rate of 1°C/min, equilibrating at 56°C for 5 min, and finally cooling to 25°C at a rate of 1°C/min. Sequences of all target oligonucleotides used are given in Supplementary Table 1.

Cloning of pRSF Z0P$_{10}$ binding site plasmid

Overlapping primers containing directly adjacent *Zif268* and *PBSII* sites with flanking HindIII restriction enzyme sites were obtained HPLC purified from IDT (Top: 5'-GGCTAGCGTGAAGCTTGCGTGGGCGGTGTGGAAAAAGCTTGCTGCGTGCC). The pRSF Z0P$_{10}$ primers were annealed at 100 μM in 1x Buffer 2 (New England Biolabs: 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$) using the procedure outlined for dsDNA production, above. Annealed pRSF Z0P$_{10}$ primers were digested with HindIII in 1x Buffer 2. Monomer *Zif268-0-PBSII* target sites were self-ligated by incubating digested DNA with T4 Ligase (New England Biolabs). These self-ligated binding sites were ligated at a molar ratio of 1:10 (vector:insert) into a pRSFDuet vector which had been cleaved with HindIII and treated with Antarctic Phosphatase (New England Biolabs). XL-1 Blue *E. coli* (Stratagene) were transformed with the purified ligation reaction. Cells were plated on LB-kanamycin and incubated at 37°C overnight. A PCR screen was used to estimate that pRSF Z0P$_{10}$ contained approximately 10 repeats of the *Zif268-0-PBSII* site. Dideoxynucleotide sequencing of the entire vector was unsuccessful due to the GC rich repeats of the ZF target sites.

Refolding protocol

All refolding experiments took place in 3.5 kDa molecular weight cut-off Slide-A-Lyzer Dialysis Cassettes (Pierce) under the conditions indicated in Supplementary Table 2. The SEER proteins of interest were mixed under denaturing conditions

(Buffer A + 4 M urea) in the presence or absence of target DNA. Refolding was induced through a dialysis procedure resulting in a final urea concentration of 350 nM. Specifically, the dialysis cassettes were placed into uncovered chambers containing 850 mL of Buffer A and maintained at 4°C for the duration of the experiment. Following 4 hours of equilibration, the buffer was exchanged with 850 mL of Buffer A. The contents of the dialysis cassette were recovered 24 hours after the initial set-up, and fluorescence readings followed directly.

Fluorescence measurements

Fluorescence readings were acquired using a Photon Technology International spectrofluorometer in conjunction with a sub-micro quartz cuvette (Starna Cells, Inc.). Excitation and emission wavelengths corresponded to accepted values for the full-length proteins: GFPuv (395/509 nm), EGFP (488/507 nm), Venus (515/528 nm), and mCherry (587/610 nm). Excitation and emission wavelengths for the mixed complementation species were determined by systematically scanning excitation and emission to identify the most intense peaks. Slit widths used for all spectra were a function of sample fluorescence intensity and were set at 5 and 8 nm (excitation) and 10 and 10 nm (emission), except for the SEER-Venus proteins (2 and 8 nm excitation slit widths). All samples were prepared in duplicate and the results represent the average of two independent measurements, normalized to the sample with the greatest fluorescence intensity, except for the pRSF $Z0P_{10}$ detection data, which is presented relative to fluorescein emission at 512 nm. Linear regressions and correlations were determined when appropriate.

Supplementary Table 1. Target oligonucleotides detected using SEER: Zif268 site is blue, PBSII site is red, and PE8B site is green. Methylation at a CpG site (orange) is indicated by "$_m$CG". The single base G to T substitution in the Zif268 site is highlighted yellow.

Target name	5'→3' (top)
Zif268-0-PBSII	GCGTAGCGTGGGCGGTGTGGAAACACCG
Zif268-0-PE8B	GCGTAGCGTGGGCGGGAGGAGATCACCG
mCpG-2-Zif268	GCGTA$_m$CGTACGCCCACGCCACCG
	CGCATGC$_m$ATGCGGGTGCGGTGGC
CpG-2-Zif268	GCGTACGTACGCCCACGCCACCG
	CGCATGCATGCGGGTGCGGTGGC
mCpG-2-Zif268 (GtoT)	GCGTA$_m$CGTACGCACACGCCACCG
	CGCATGC$_m$ATGCGTGTGCGGTGGC
mCpG only	GCGTA$_m$CGTAGCACATAGGCACCG
	CGCATGC$_m$ATCGTGTATCCGTGGC

Supplementary Table 2. Refolding concentrations corresponding to the experiments described in the main text and supplementary information.

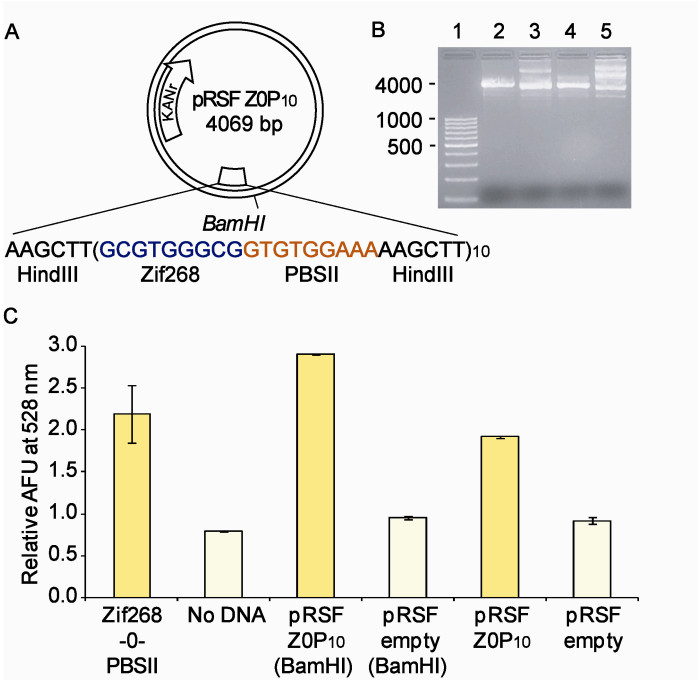
Figure	SEER system	[NFP-X]/μM[a]	[Y-CFP]/μM[a]	[target]/nM
2	SEER-GFPuv	0.67	5.33	250 – 10[b]
2	SEER-EGFP	0.50	1.00	250 – 10[b]
2	SEER-Venus	0.67	1.33	250 – 10[b]
2	SEER-mCherry	8.00	2.00	250 – 10[b]
2, bottom left	mCpG-SEER-Venus	0.50	2.00	250
2, bottom right	mCpG-SEER-Venus	0.50	2.00	250 - 25[b]
3B	SEER-Venus/GFPuv	1.34	1.34, each	150, each[c]
3D	SEER-Venus/Cerulean	1.34	1.34, each	150, each[c]
S1C	SEER-Venus	0.67	1.34	100, *Zif268-0-PBSII* 10, pRSF $Z0P_{10}$

[a] Systematically optimized for maximum signal above background.

[b] Titration.

[c] Detection of a single oligonucleotide at 150 nM or of two targets, each present at 150 nM.

Supplementary Figure 1. *Zif268-0-PBSII* binding site plasmid. (A) pRSF $Z0P_{10}$ contains ten *Zif268-0-PBSII* binding sites, each separated by a HindIII restriction site. (B) Agarose gel analysis of pRSF $Z0P_{10}$ and pRSF empty following BamHI cleavage to relieve supercoiling. Lane 1: Size markers (bp); Lane 2: pRSF $Z0P_{10}$ (BamHI), Lane 3: pRSF $Z0P_{10}$; Lane 4: pRSF empty (BamHI); Lane 5: pRSF empty. (C) NVenus-Zif268 and CVenus-PBSII were mixed in the presence or absence of *Zif268-0-PBSII* or in the presence of pRSF $Z0P_{10}$ or pRSF empty, which were either supercoiled or linearized with a BamHI digestion. Samples were excited at 515 nm, and emission was collected at 528 nm.


A
pRSF Z0P10
4069 bp
KANr
BamHI
AAGCTT(GCGTGGGCGGTGTGGAAAAAGCTT)10
HindIII
Zif268
PBSII
HindIII
B
1 2 3 4 5
4000
1000
500
C
Relative AFU at 528 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Zif268 -0- PBSII
No DNA
pRSF Z0P10 (BamHI)
pRSF empty (BamHI)
pRSF Z0P10
pRSF empty