http://dx.doi.org/10.5012/bkcs.2014.35.5.1455

FRET-Based Quantitative Discrimination of Bisulfite-Untreated DNA from Bisulfite-Treated DNA

Eun Jeong Lee, Yea Seul Cho, Seongeun Song, Sang-Hyun Hwang,[†,‡,*] and Sang Soo Hah[*]

Department of Chemistry and Research Institute for Basic Sciences, Kyung Hee University, Seoul 131-701, Korea
[*]E-mail: sshah@khu.ac.kr
[†]Department of Laboratory Medicine, Center for Diagnostic Oncology, Research Institute and Hospital, National Cancer Center, Goyang-si, Gyeinggi-do 410-769, Korea
[‡]Hematologic Malignancy Branch, Research Institute and Hospital, National Cancer Center, Goyang-si, Gyeinggi-do 410-769, Korea. [*]E-mail: mindcatch@ncc.re.kr
Received January 13, 2014, Accepted January 22, 2014

We report a sensitive and reliable FRET-based nanotechnology assay for efficient detection and quantification of bisulfite-unmodified or modified DNA. Bisulfite-untreated DNA or bisulfite-treated DNA is subjected to PCR amplification with biotin-conjugated primers so that the amounts of bisulfite-untreated and treated DNA can be differentiated. Streptavidin-coated quantum dots (QDs) are used to capture biotinylated PCR products intercalated with SYBR Green, enabling FRET measurement. Key features of our method include its low intrinsic background noise, high resolution, and high sensitivity, enabling detection of as little as 1.75 ng of bisulfite-untreated DNA in the presence of an approximately 1,000-fold excess of bisulfite-untreated DNA compared to bisulfate-treated DNA, with the use of PCR reduced (as low as 15 cycles). SYBR Green as an intercalating dye as well as a FRET acceptor allows for a single-step preparation without the need for primers or probes to be chemically conjugated to an organic fluorophore. Multiple acceptors per FRET donor significantly enhance the signal-to-noise ratio as well. In consideration of the high relevance of bisulfite treatment to DNA methylation quantitation, our system for FRET measurement between QDs and intercalating dyes can be generally utilized to analyze DNA methylation and to potentially benefit the scientific and clinical community.

Key Words : FRET, DNA methylation, Bisulfite treatment, dsDNA intercalating dye

Introduction

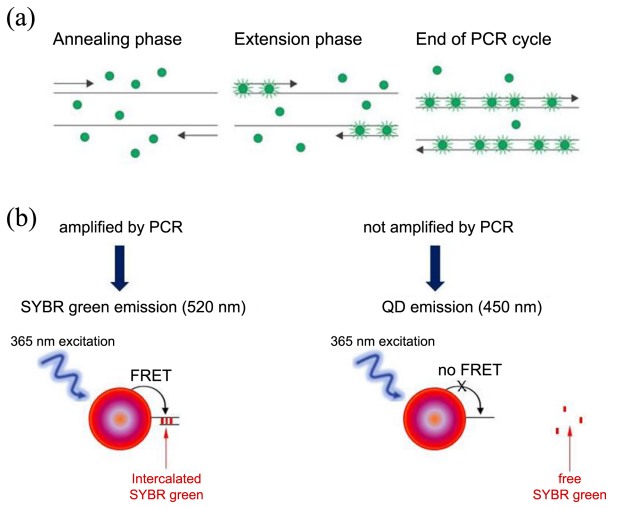
DNA methylation is an epigenetic modification that occurs at cytosine in CpG dinucleotides and is usually observed in high frequency in the promoter regions of tumor-suppressor genes.[1,2] Such modifications account for the loss of function of tumor-suppressor genes for almost every type of cancer,[3] and more genes are known to be inactivated by DNA methylation or hypermethylation in the promoter regions than by genetic mutations.[4] These aberrant epigenetic changes appear to be an early event that precedes the occurrence of genetic mutations.[5,6] In this respect, quantitation of DNA methylation in the promoter regions is a valuable tool for early diagnosis of cancer, tumor behavior monitoring, and response measurement for targeted therapy.[7,8]

A number of tools available to assess DNA methylation demonstrate the extensive interest that has been invested in understanding the role of epigenetics in carcinogenesis, and methylation-specific PCR (MSP) is one of the most commonly used methods for the detection of DNA methylation.[9] Of importance, this technique relies on sodium bisulfite treatment (or simply bisulfite treatment) of DNA, which was discovered by Hayatsu *et al.* in 1970.[10] Bisulfite treatment of DNA converts unmethylated cytosines to uracils while leaving methylated cytosines unaffected (Fig. S1 in the supplementary material).[10] The converted sequences are then able to be amplified with specific primers, and the amplified products are identified using gel electrophoresis. However, this standard MSP method is limited to qualitative analysis. Although real-time PCR-based MSP methods[11,12] using double-stranded (ds) DNA-specific intercalating dyes, followed by and C_T (cycle threshold) measurement, allow for quantitative analysis, they exhibit low sensitivity for direct screening of challenging samples, where the amount of DNA is minimal, thus requiring a nested PCR approach (see Fig. S2, S3, and S4).[13-16]

To overcome some disadvantages of the conventional DNA methylation quantitation methods based on bisulfite treatment of DNA, several quantitative detection of DNA methylation based on fluorescence resonance energy transfer (FRET) between quantum dots (QDs) and fluorogenic dyes, such as Cy5, have been recently developed.[17] In general, FRET is a state-to-state energy transfer between the excited and ground states of the fluorogenic dyes, in which the excitation energy is transferred from a donor to an acceptor without the emission of a photon, with inverse sixth-power dependence on the distance between the two dyes.[18] The efficiency of energy transfer is high when the emission spectrum of the donor substantially overlaps with the absorption spectrum of the acceptor and when the distance between

them is 20–100 Å.[18] At an optimal distance, emission from the donor is suppressed and emission from the acceptor is enhanced, leading to a shift in the color of the emitted light toward light of a longer wavelength.[18] Such energy transfer processes could provide us with significant information on the distance and interaction between two dyes, based on the intensities and wavelength shifts of the absorption and emission.[18] Notably when QDs are used as FRET donors, the large Stokes shift and narrow emission spectra of QDs allow for FRET with a significantly reduced acceptor excitation and spectral crosstalk, with the minimal background necessary for detecting targets at low-concentration.[17,19] Especially, Bailey *et al.* reported that this feature of low intrinsic background of QD-based FRET systems facilitated the development of a methylation-detection method with greater sensitivity than the conventional MSP methods, as exemplified by analyzing sputum samples from lung cancer patients.[17]

Inspired by the recent work by Bailey *et al.*,[17] we designed a new quantitative assay in the sense that FRET acceptors could be non-covalently introduced, for bisulfite treatment of DNA based on FRET between QDs as FRET donors and SYBR Green as intercalating dyes as well as FRET acceptors, where the high specificity of MSP could be combined with the high sensitivity and simplicity of the quantum dot-based FRET (QD-based FRET) technology.[17,19]



Scheme 1. Principle of QD-based FRET for DNA quantitation. (a) Intercalating dyes (SYBR Green in this study) during PCR amplification. It should be noted that intercalated SYBR Green can fluoresce at 520 nm when excited at 490 nm, but cannot when excited at 365 nm. (b) Quantitation of PCR products based on FRET measurement between QDs and SYBR Green. For DNA methylation quantitation, DNA templates can be subjected to sodium bisulfite conversion (see Fig. S1 in the supplementary material), wherein unmethylated cytosines can be converted to uracil while methylated cytosines remain unaffected. The resulting DNA or the original DNA template is amplified using PCR in the presence of either one of the two biotin-labeled primer sets (*i.e.* bisulfite-untreated DNA-specific primers and bisulfite-treated DNA-specific primers, see Fig. S2) and of SYBR Green. The resulting SYBR Green-intercalated and biotin-conjugated PCR product is bound to streptavidin-coated QDs. And finally, the nanoassembly when excited at 365 nm allows for FRET to occur between the QD donor and the fluorophore acceptor, which can be used for DNA quantitation.

As Scheme 1 highlights the principle of the method, a synthesized DNA target with a sequence of *hMLH1* gene promoter[20] (37034875-37035945) is first modified with sodium bisulfite to convert unmethylated cytosines to uracil, leaving the methylated cytosines unconverted (Fig. S1). Subsequently, DNA enzymatic replication or PCR in the presence of either one of the two primer sets (*i.e.* bisulfite-untreated DNA-specific primers and bisulfite-treated DNA-specific primers) is used to amplify the bisulfite-untreated or bisulfite-treated DNA (Fig. S2), of which the concentration of the amplified DNA can be estimated by dsDNA-specific intercalating dyes. We specifically used SYBR Green that serves as a dsDNA intercalating dye (Scheme 1(a)) as well as a FRET acceptor while streptavidin-coated QD as a FRET donor. The primers of each primer set used in the replication were conjugated with biotin at 5'-end so that the amplified products could be bound to streptavidin-coated QDs and that the presence of the amplified DNA could be precisely quantified with high sensitivity by FRET between QD and SYBR Green (Scheme 1(b)).

Experiments

Chemicals and Materials. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. QDs with emission maxima of 450 nm and modified with carboxylic acid groups were obtained from Ocean Nanotech and the QD concentrations were measured by optical absorbance, using extinction coefficients provided by the supplier. Conjugation with streptavidin was carried out according to the manufacturer's protocol. Fluorescence spectra were recorded using a Synergy Mx spectrofluorophotometer (BioTek). All experiments were performed in triplicate.

Bisulfite Modification and PCR. HPLC-purified DNA templates having a sequence of *hMLH1* gene promoter (37034875-37035945, 5'-GAC TGG CAT TCA AGC TGT CCA ATC AAT AGC TGA CGC TGA AGG GTG GGG CTG GAT GGC GTA AGC TAC AGC T-3') and biotin-conjugated primers (primers A and B for bisulfite-untreated DNA template: 5'-biotin-AGC TGT AGC TTA CGC CAT CCA-3' and 5'-biotin-GAC TGG CAT TCA AGC TGT CCA-3', respectively; primers C and D for bisulfite-treated DNA template: 5'-biotin-AAC TAT AAC TTA CAC CAT CCA-3' and 5'-biotin-AAC TAA CAT TCA AAC AAT CCA-3', respectively) were obtained from Bioneer (Daejeon, Korea). DNA bisulfite modification was performed according to the manufacturer's protocol (EZ DNA Methylation-Gold kit, Zymoresearch). PCR in the presence of SYBR Green (2×PreMix Solution, Enzynomics) was run using RealTime PCR FQD-48A (BIOER) with DNA template concentrations varied and primer concentrations fixed at 0.2 mM, of which the PCR running conditions were 95 °C for 15 min, followed by 35 or 15 cycles of 30 sec at 94 °C, 30 sec 51 °C sec and 40 sec at 72 °C, with a final extension

cycle of 72 °C for 2 min. The concentration of the amplified DNA in the middle of PCR amplification was estimated by fluorescence measurement resulting from intercalated SYBR Green (excitation at 490 nm, emission at 520 nm) and C_T values were obtained according to the manufacturer's protocol. Products were subjected to PCR purification (QIAquick PCR Purification Kit or Illustra MicroSpin G-50 (or G-25) Columns (GE Healthcare). Final streptavidin-coated QD concentration was 0.1 nM.

QD-based FRET Detection of Amplified DNA. Initial amounts of bisulfite-untreated or treated DNA template for PCR amplification range from 1.75 ng to 3.5 μg, and mixtures of bisulfite-untreated or –treated DNA template for PCR amplification range from 0% to 100% of the total 1 μg of bisulfite-untreated and treated DNA. The template mixture was used for 15-cycle PCR reaction in the presence of primers A and B or primers C and D. To precisely quantify the level of amplified DNA, we measured the ratios between the fluorescence intensities at 520 nm and at 450 nm for the normalized FRET efficiencies in our system, because as the level of the acceptor emission increases, the decay of donor emission increases as well in any FRET process.

Results and Discussion

FRET Events Between QDs and SYBR Green and FRET-Based Quantitation of DNA. Like the conventional MSP-based methods,[11,12] the bisulfite-untreated DNA and the bisulfite-treated DNA in our system are able to be amplified through PCR in the presence of primers A and B and of primers C and D, respectively (Fig. S2). Because the primers are biotinylated and the amplified DNAs are intercalated with fluorophores SYBR Green, streptavidin-conjugated quantum dots introduced after PCR amplification, are able to capture the PCR products *via* streptavidin-biotin binding, bringing the QDs (serving as FRET donors) and SYBR Green (serving as FRET acceptors) in close proximity allowing FRET to occur. Finally, PCR products obtained from 15-cycle PCR reactions are detected by the relative fluorescence intensities (F_{520}/F_{450}) caused by FRET between QDs and intercalating dyes, which can be used for quantitation of initial amounts of DNA templates.

To validate the proof-of-concept, PCR experiments using a synthesized DNA target with a sequence of *hMLH1* gene promoter before or after bisulfite treatment were conducted in the presence of bisulfite-untreated or treated DNA-specific primers labeled with biotin at 5'-termini (primers A and B or primers C and D) and of dsDNA-specific intercalating dye, SYBR Green, which was then followed by incubation with streptavidin-coated QDs. As observed in Figure 1(a), FRET signals confirm the presence of amplified targets with SYBR Green intercalated. Detection was possible even after a single enzymatic replication, and FRET efficiency increased with replication cycles (data not shown). Figure 1(b) demonstrates that after PCR experiments in the presence of primers A and B, initial amounts of bisulfite-untreated DNA template have linear relationship with the relative fluorescence intensities (F_{520}/F_{450}) caused by FRET between QDs and intercalating dyes, which is different from the relationship between initial amounts of bisulfite-untreated DNA template and the C_T values (Fig. S4). While the C_T values as a function of initial amount of DNA show a saturation curve, our method based on FRET between QDs and intercalating dyes exhibits clear linearity. It should be noted that because PCR experiments with the bisulfite-untreated DNA templates in the presence of primers C and D or with bisulfite-treated DNA templates in the presence of primers A and B cannot amplify the templates (Fig. S2 and S4), no significant fluorescence intensities (F_{520}/F_{450}) are able to be obtained.

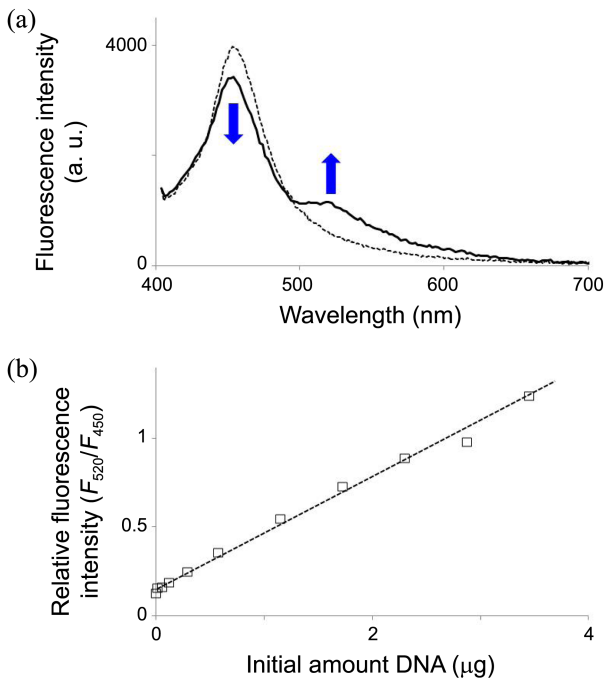


Figure 1. (a) Fluorescence spectra changes of streptavidin-coated QDs (dotted line) and the nanoassembly of streptavidin-coated QDs (1 pmol) bound to SYBR Green-intercalated and biotin-conjugated PCR product (0.55 μg) (solid line), showing that the binding event produces a significant FRET signal change for sensitive DNA quantitation. B. DNA Quantitation based on FRET between QDs and intercalating dyes. After 15-cycle PCR experiments for bisulfite-untreated DNA templates in the presence of primers A and B, the relative fluorescence intensities (F_{520}/F_{450}) caused by FRET between QDs and intercalating dyes are plotted as a function of initial amounts of bisulfite-untreated DNA template, clearly showing a linear relationship in a wide range from 1.75 ng to 3.5 μg of DNA, different from the relationship between C_T values and initial amounts of DNA (see Fig. S4). An $R^2 = 0.99$ was obtained for the fitting line. It should be noted that because PCR experiments with the bisulfite-untreated DNA templates in the presence of primers C and D or with bisulfite-treated DNA templates in the presence of primers A and B cannot amplify the templates, no significant fluorescence intensities (F_{520}/F_{450}) can be obtained.

Most QD-based FRET assays for DNA or RNA sensing usually utilize streptavidin-coated QDs because of the robust binding between streptavidin and biotin, and of the negatively charged surfaces, which restrict nonspecific DNA/RNA

binding.[17,19,21,22] Importantly, however, singly terminally labeled probes have been used for FRET detection driven by specific hybridization the targets of interest. As a result, the long separation distance from QDs to acceptors in the assembly limits FRET efficiency. Since the nucleic acid sizes vary from gene to gene and FRET efficiency depends on the distance between the FRET pair, this could impose a constraint on the FRET analysis. Increasing FRET efficiency, and thereby improving detection sensitivity, can be facilitated by increasing the number of FRET acceptors per QD. In this respect, our method is advantageous over the conventional QD-based FRET assays for DNA or RNA sensing. To our best knowledge, this is the first demonstration of a QD-FRET-based assay for sensing DNA wherein multiple SYBR Green as FRET acceptors are intercalated, which enhances the FRET efficiency and detection sensitivity. The results shown in Figure 1(b) demonstrate successful detection of as little as 1.75 ng of DNA, thereby illustrating the ultrasensitive capability of our method. It should also be noted that larger DNA templates could result in stronger FRET signals due to the larger amounts of SYBR Green intercalated.

Quantitation of DNA Based on Primer-specific PCR Amplification and FRET Between QDs and SYBR Green. Very interestingly, there is a corresponding linear increase in the relative fluorescence intensities (F_{520}/F_{450}) caused by FRET, when the amount of bisulfite-untreated DNA in the mixture is increased (with a fixed total DNA amount of 1 μg) and almost exactly *vice versa*, as shown in Figure 2. The two lines are crossed when the amounts of two DNA templates are approximately equal, of which the relative fluorescence intensity (F_{520}/F_{450}) is half of the sum of the maximum and minimum values, suggesting that primers A and B can work for bisulfite-untreated DNA and primers C and D for bisulfite-treated DNA and that the exact amount of initial bisulfite-treated DNA template can be quantitated in comparison to the exact amount of initial bisulfite-treated DNA template. Scheme 2 illustrates the importance of these results, implying that our method can be employed for future DNA methylation quantitation assays because spectral information can be processed to determine the level of DNA methylations; for example, when one wants to quantify the methylation of 1 μg of *hMLH1* gene promoter, two sets of bisulfite-untreated and treated DNA templates are amplified by PCR in the presence of bisulfite-untreated or treated DNA-specific primers labeled with biotin at 5'-termini (primers A and B or primers C and D, please see Fig. S2), and the resulting PCR products are bound to streptavidin-coated QDs for FRET measurement and thus for DNA methylation quantitation, because 1) the total amount of DNA can be assayed by PCR in the presence of primers A and B before bisulfite treatment (① and ⑤ in Fig. S2, and thick arrow in Scheme 2), followed by FRET measurement, 2) methylated DNA can be assayed by PCR in the presence of primers A and B after bisulfite treatment (② and ⑥ in Fig. S2, and thin arrow in Scheme 2), and finally, 3) unmethylated DNA can be quantified, as a double check, when the template is amplified in the presence of primers C and D after bisulfite treatment (④ and ⑧ in Fig. S2, and thin arrow in Scheme 2. Fig. S2 suggests that other combinations are also possible in principle.

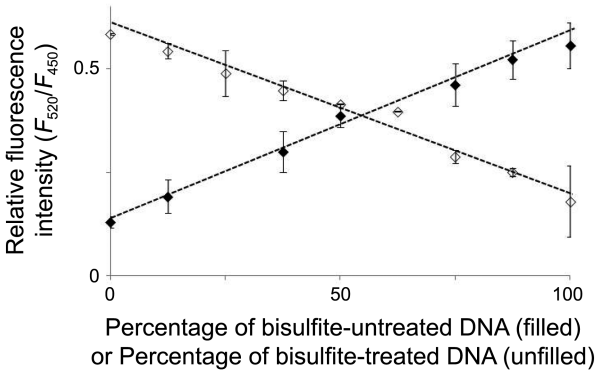
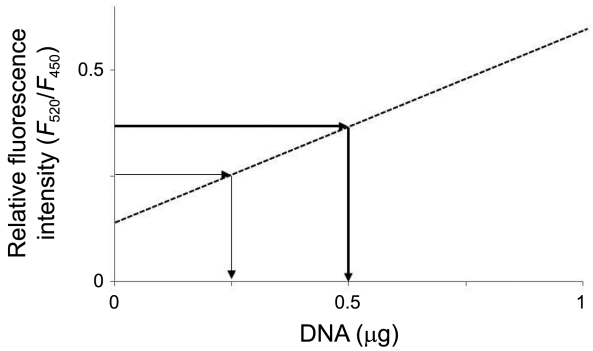


Figure 2. DNA Quantitation based both on primer-specific PCR amplification and FRET between QDs and intercalating dyes. After 15-cycle PCR experiments for bisulfite-untreated DNA templates in the presence of primers A and B (filled) or for bisulfite-treated DNA templates in the presence of primers C and D (unfilled), the relative fluorescence intensities (F_{520}/F_{450}) caused by FRET between QDs and intercalating dyes are plotted as a function of the amount percentage of bisulfite-untreated DNA in the mixture (with a fixed total DNA amount of 1 μg, because the standard protocol for bisulfite treatment usually uses 0.5 μg of DNA). The two lines are crossed when the amounts of two DNA templates are approximately equal, of which the relative fluorescence intensity (F_{520}/F_{450}) is half of the sum of the maximum and minimum values.



Scheme 2. Principle of DNA methylation quantitation based on FRET measurement between QDs and intercalating dyes. The described method can be employed for future DNA methylation quantitation assays because spectral information can be processed to quantitatively determine the total amount of DNA assayed and the level of DNA methylation.

Conclusions

To summarize, we report a robust, sensitive QD-based system that is capable of quantitative discrimination between bisulfite-untreated and treated DNA by FRET measurement between QDs and dsDNA-specific intercalating dyes. SYBR Green as an intercalating dye as well as a FRET acceptor allows for a single-step preparation without the need for primers or probes to be chemically conjugated to an organic fluorophore. Multiple acceptors per FRET donor significant-

ly enhance the signal-to-noise ratio, and therefore increase the overall sensitivity of the FRET system. The large dynamic range of the QD system may enable analysis of challenging clinical samples wherein the target DNA concentration is unknown. In consideration of the high relevance of bisulfite treatment to DNA methylation quantitation, our system for FRET measurement between QDs and intercalating dyes can be generally utilized to analyze DNA methylation and to potentially benefit the scientific and clinical community.

Acknowledgments. This work was supported by a grant from the Kyung Hee University in 2013 (KHU-20130549).

References

1. Jones, P. A.; Baylin, S. B. *Cell* **2007**, *128*, 683-692.
2. Herman, J. G.; Baylin, S. B. *N. Engl. J. Med.* **2003**, *349*, 2042-2054.
3. Holliday, R.; Grigg, G. W. *Mutat. Res.* **1993**, *285*, 61-67.
4. Schuebel, K. E.; Chen, W.; Cope, L.; Glcckner, S. C.; Suzuki, H.; Yi, J. M.; Chan, T. A.; van Neste, L.; van Criekinge, W.; van den Bosch, S.; van Engeland, M.; Ting, A. H.; Jair, K.; Yu, W.; Toyota, M.; Imai, K.; Ahuja, N.; Herman, J. G.; Baylin, S. B. *PLoS Genet.* **2007**, *3*, 1709-1723.
5. Bird, A. *Genes Dev.* **2002**, *16*, 6-21.
6. Russo, V. E. A.; Martienssen, R. A.; Riggs, A. D. *Epigenetic Mechanisms of Gene Regulation*; Cold Spring Harbor Laboratory Press: New York, 1996.
7. Esteller, M.; Garcia-Foncillas, J.; Andion, E.; Goodman, S. N.; Hidalgo, O. F.; Vanaclocha, V.; Baylin, S. B.; Herman, J. G. *N. Engl. J. Med.* **2000**, *343*, 1350-1354.
8. Brock, M. V.; Hooker, C. M.; Ota-Machida, E.; Han, Y.; Guo, M.; Ames, S.; Gloeckner, S.; Piantadosi, S.; Gabrielson, E.; Pridham, G.; Pelosky, K.; Belinsky, S. A.; Yang, S. C.; Baylin, S. B.; Herman, J. G. *N. Engl. J. Med.* **2008**, *358*, 1118-1128.
9. Laird, P. W. *Nat. Rev. Cancer* **2003**, *3*, 253-266.
10. Hayatsu, H.; Wataya, Y.; Kai, K.; Iida, S. *Biochemistry* **1970**, *9*, 2858-2865.
11. Lo, Y. M.; Wong, I. H.; Zhang, J.; Tein, M. S.; Ng, M. H.; Hjelm, N. M. *Cancer Res.* **1999**, *59*, 3899-3903.
12. Eads, C. A.; Danenberg, K. D.; Kawakami, K.; Saltz, L. B.; Blake, C.; Shibata, D.; Danenberg, P. V.; Laird, P. W. *Nucleic Acids Res.* **2000**, *28*, e32.
13. Brandes, J. C.; van Engeland, M.; Wouters, K. A.; Weijenberg, M. P.; Herman, J. G. *Carcinogenesis* **2005**, *26*, 1152-1156.
14. Belinsky, S. A.; Liechty, K. C.; Gentry, F. D.; Wolf, H. J.; Rogers, J.; Vu, K.; Haney, J.; Kennedy, T. C.; Hirsch, F. R.; Miller, Y.; Franklin, W. A.; Herman, J. G.; Baylin, S. B.; Bunn, P. A.; Byers, T. *Cancer Res.* **2006**, *66*, 3338-3344.
15. Machida, E. O.; Brock, M. V.; Hooker, C. M.; Nakayama, J.; Ishida, A.; Amano, J.; Picchi, M. A.; Belinsky, S. A.; Herman, J. G.; Taniguchi, S.; Baylin, S. B. *Cancer Res.* **2006**, *66*, 6210-6218.
16. Kim, D. S.; Kim, M. J.; Lee, J. Y.; Kim, Y. Z.; Kim, E. J.; Park, J. Y. *Cancer* **2007**, *110*, 2785-2792.
17. Bailey, V. J.; Easwaran, H.; Zhang, Y.; Griffiths, E.; Belinsky, S. A.; Herman, J. G.; Baylin, S. B.; Carraway, H. E.; Wang, T.-H. *Genome Res.* **2009**, *19*, 1455-1461.
18. Valeur, B. *Molecular Fluorescence: Principles and Applications*; Wiley-VCH: Weinheim, Germay, 2002.
19. Zhang, C.-Y.; Yeh, H.-C.; Kuroki, M. T.; Wang, T.-H. *Nat. Mater.* **2005**, *4*, 826-831.
20. Barakat, R.; Berchuck, A.; Markman, M.; Randall, M. E. *Principles and Practice of Gynecologic Oncology*; Lippincott Williams & Wilkins: Wolters Kluwer Health, Philadelphia, USA, 2013.
21. Zhang, C.-Y.; Johnson, L.W. *J. Am. Chem. Soc.* **2006**, *128*, 5324-5325.
22. Hohng, S.; Ha, T. *ChemPhysChem* **2005**, *6*, 956-960.