IDENTIFICATION OF SINGLE MOLECULAR DNA METHYLATION POINTS BY MICROFLUIDIC DNA MOLECULE STRETCHING AND QUANTUM DOT DETECTION

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ABSTRACT

We report a rapid and simple epigenetic analysis based on microfluidic single DNA molecular methylation detection. We developed a microfluidic device to stretch a single DNA molecule and subsequent detection of a methylated DNA base by quantum dot (QD)-immobilized methyl binding domain (MBD) protein. A QD-MBD complex specifically bound to a methylated base of stretched DNA and was detected by a total internal reflection fluorescence microscope (TIRFM). Our method enables us to detect a number of methylation site and identify the methylated positions simultaneously in the simple and rapid manner compared with the conventional methods.

KEYWORDS

Single DNA molecule analysis, Epigenetic analysis, Microfluidic device, Quantum dot

INTRODUCTION

Epigenetics, such as DNA methylation, is becoming important in the screening of the early stage of cancer. For DNA methylation analysis, immunoprecipitation using methylcytosine antibody, bisulfite sequencing *etc* are employed currently. However, they require a long reaction time, large sample volume and troublesome procedures, and cause cleavage of DNA. Recently Cipriany *et al*. reported high-throughput detection and analysis of DNA methylation using nanofluidic channels. However, this method cannot identify where and how many sites of DNA is methylated. Recently, we developed the technique to observe single stretching DNA molecule [1-4]. In this paper, to overcome present problems of methylation analysis, we applied our technique to single DNA molecule methylation analysis.

PRINCIPLE

The schematic illustration of single DNA molecule methylation analysis method is shown in Figure 1. DNA was ligated with complementary biotinylated oligonucleotide and immobilized on the glass surface via biotin-streptavidin complex formation. Subsequently, QD-MBD complexes were injected into the microchannel, bound to methylation site under DNA stretching, and detected with a total internal reflection fluorescence microscope.

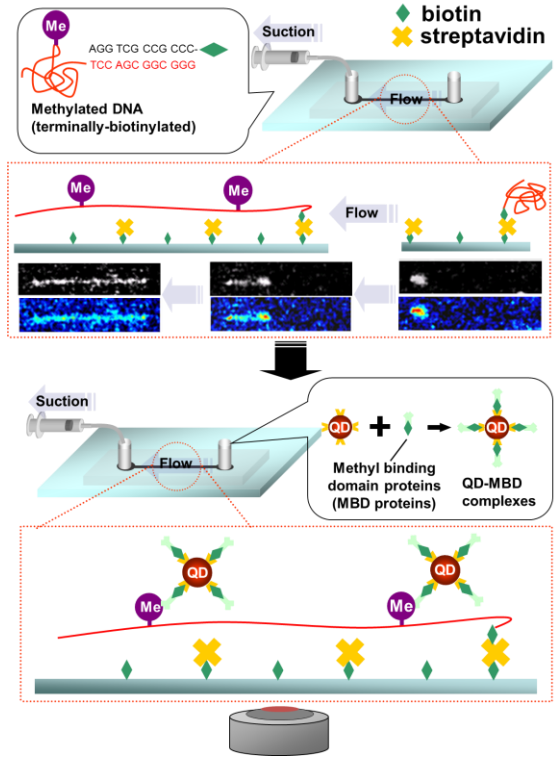

Figure 1. The scheme of single DNA molecule methylation analysis method.

EXPERIMENTAL

Employed microfluidic device shown in Figure 2(a) was prepared according to previous reported papers [1-4]. Briefly, poly(dimethyl siloxane) (PDMS) microchannel (265 μm width, 50 μm height) was fabricated by the soft lithography method and bonded with a cover glass physically coated with biotin-bovine serum albumin (BSA) and streptavidin. QD-MBD complexes were prepared by mixing streptavidin coated QD and biotin-MBD. To estimate the optimum mixing ratio, we attempted to purify QD-MBD complexes from reactants by two methods. One is the ultrafiltration method and the other is the magnetic beads method. In magnetic beads method, streptavidin coated magnetic beads were mixed into QD-MBD reaction solution to bind unreacted MBD proteins and recovered only QD-MBD complexes using neodymium magnet after 10 min incubation. To distinguish the fluorescence of QD from that of YOYO-1, construction of the detection system was attempted by using the two types of fluorescence mirror unit, U-MWIB3 (BP 460-490 nm, BA 510nm, OLYMPUS) and U-MF2 (BP460-490 nm, BA 580 nm, OLYMPUS), respectively. Using U-MWIB3, both fluorescence of QD and YOYO-1

were detected. On the other hand, using U-MF2, only fluorescence of QD was detected. To detect DNA methylation, λ-DNA was methylated by BamH1 methyltransferase (Recognition sequence: 5'-GGATCC-3', the number of methylation sites were 5) and terminally biotinylated. And then, methylated DNA was introduced into the streptavidin coated microchannel. After 10 min incubation, not-immobilized DNA was washed with ultrapure water. Then, QD-MBD complexes were introduced into the microchannel, incubated for 10 min, and washed with ultrapure water. Lastly, single molecule observation was attempted at the flow rate of 1 μl/min with TIRFM.

RESULTS AND DISCUSSION

The fluorescence image of stretching λ-DNA molecules and the histogram of λ-DNA length in Figure 2(b) and (c) indicate successful stretching of λ-DNA with our device. In addition, our method can preserve native DNA length without cleavage of DNA as shown in Figure 2(c) and (d). Secondly, to confirm the preparation of QD-MBD complex, gel shift assay and measurement of zeta potential of each samples prepared by different mixing ratio were performed as shown in Figure 3. Figure 3(a) and (b) show that sample 3 and 4 have similar zeta potential and higher one compared to native QD and sample 1, which mean in sample 3 and 4, QD completely bound MBD proteins via biotin-streptavidin interaction and excess unbounded MBD proteins are remained. To remove the excess MBD, purification was attempted and confirmed with gel shift assay as shown in Figure 3(c). Figure 3(c) indicates that employment of streptavidin coated magnetic beads can successfully remove unbounded MBD proteins from reactants. Subsequently, the construction of detection system was attempted. Figure 4 (b)-1and -2 show the fluorescence images of stretching DNA by molecular combing stained with YOYO-1 and QD through (a)U-MWIB3 and (b) U-MF2. When

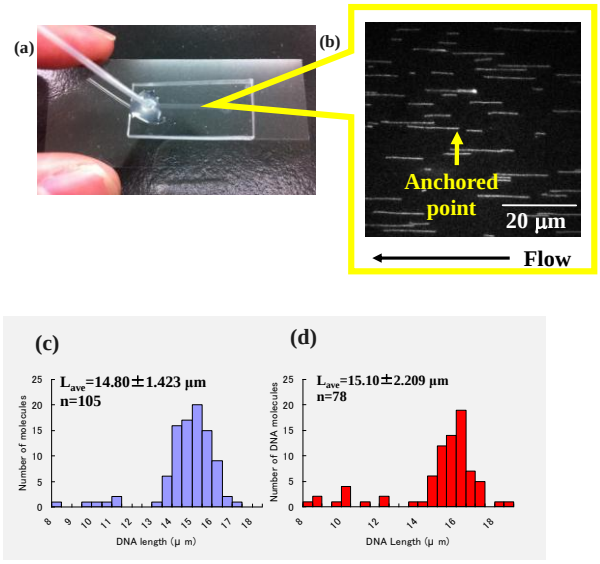

Figure 2. (a)A photograph image of our microdevice, which width and depth were 1000 μm and 50 μm, respectively. (b)a fluorescence microscopy image of stretching DNA molecules. The histogram of (c) λDNA length and (d)methylated λDNA treated by BamHI methyltransferase

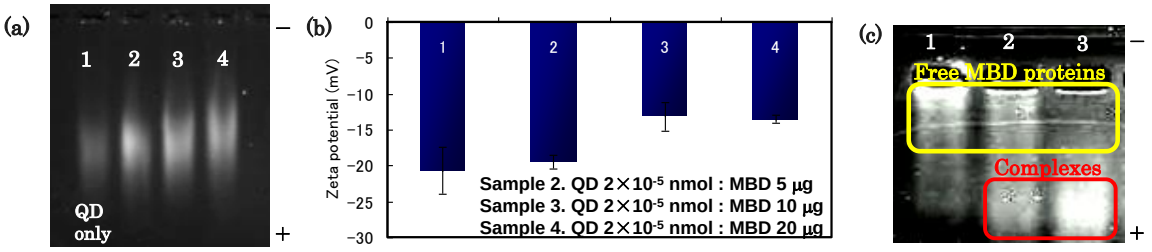

Figure 3. Confirmation of QD-MBD complexes by the (a) Gel shift assay and (b)the measurement of zeta potential. Lane1, QD only; Lane 2-4 QD-MBD complexes. (c)Confirmation of purification by gel electrophoresis Lane 1, only MBD proteins; lane 2, ultrafiltration; lane 3, magnetic beads method.

QDs and stretching DNA stained with YOYO-1 were observed through U-MF2, the only fluorescence signal of YOYO-1 disappeared, while the fluorescence of QDs was nearly-unchanged compared with that through U-MWIB3. This result indicates that employment of appropriate filter can permit to distinguish fluorescence from them. Finally, the detection of DNA methylation was attempted. Figure 4 (c) shows five fluorescence dots were observed from stretched methylated single λDNA, which is also confirmed with fluorescence from YOYO-1 as shown in Figure 4(b). Furthermore, the distance between adjacent fluorescence dots were measured and compared with predicted one as shown in Figure 4(a). Table 1 shows that measured distance well corresponded with calculated and predicted one. From these results, we demonstrate we can simultaneously detect DNA methylation, and analyze the number and the location of methylation sites in the simple and rapid manner.

Thus, our developed method can permit simpler and more rapid methylation analysis compared to present method. Therefore, our method could be applied for diagnosis of early stage of cancer

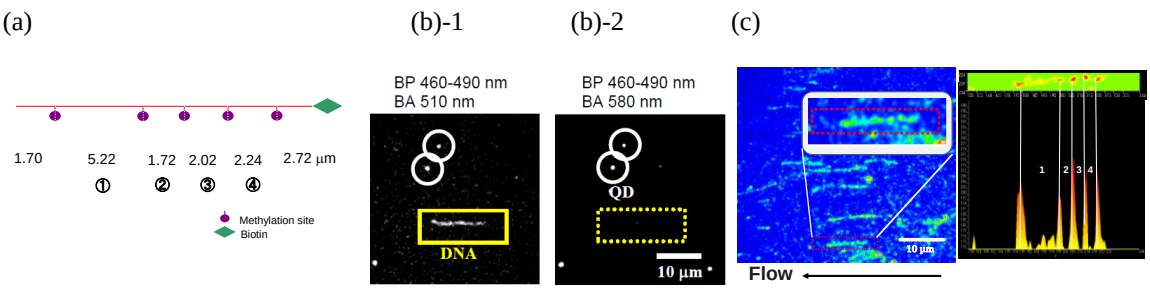

Figure 4 (a)methylation sites in λDNA sequence and the distance between methylation sites.

(b) Fluorescence images of YOYO-1 stained λDNA and QD with two type filter.

(c) Fluorescence image of QD-MBD complexes binding to stretched methylated λ DNA and histogram of five fluorescence dots

Table 1. Distance between adjacent methylation point

Relative distance (calculated value)	0.45	0.16	0.18	0.2
Relative distance (measured value)	0.50	0.19	0.15	0.15

CONCLUSION

We developed a rapid and simple epigenetic analysis method based on microfluidic single DNA molecular methylation detection. Our developed method can overcome present problems related to methylation analysis and attain high performance in epigenetic analysis. Therefore, our method could be applied for diagnosis of early stage of cancer in clinic.

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