Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Peroxotungstate Oxidation-Mediated Two-Phase Amplification System (POM-TPAS) for Bisulfite-Free Quantification of Locus-Specific 5-Hydroxymethylcytosine

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1. Reagents and Materials

dATP, dTTP, dCTP, dGTP (100 mM each), Dr.GenTLE® Precipitation Carrier and nuclease-free water are purchased from TaKaRa (Dalian, China). JumpStart™ Taq DNA polymerase, formamide and potassium tungstate are ordered from Sigma-Aldrich (Saint Louis, USA). The Streptavidin Mag Sepharose microbeads, which are composed of iron oxide cores embedded in agarose matrices and are surface-functionalized with streptavidin (Stv), are purchased from GE Healthcare (Uppsala, Sweden). The biotin-dATP, Alexa Fluor 546-Stv conjugates are supplied by Life Technologies (Carlsbad, CA, USA). All oligonucleotides (Table S1) containing modified bases are synthesized and purified by TaKaRa Biotechnology Co., Ltd (Dalian, China). All of other chemical reagents are analytical grade and used without further purification.

Table S1. The DNA sequences used in this study.

Name	Sequence (5'-3')
ProbeB-16base	Double-biotin-TTTTTTTTTTTTTTTTTTTTGGGATACTGGAACCTGATGATGACTAATACCTGCGTGATAAGTTTGAAGAGCCCCA
ProbeA-18base	PO_4-GGTGATAGATCTGTGGGATTTACTACTCTGACTACCTCTATGCTTGCTACCGTCG
Reverse primer	CGACGGTAGCAAGCATAGAGGTAG
Forward primer	GGGATACTGGAACCTGATGATGAC
Target C	CCAGGTCCCACAGATCTATCACCCGGGGCTCTTCAAACTCTGCAGG
Target 5mC	CCAGGTCCCACAGATCTATCACC^{5m}CGGGGCTCTTCAAACTCTGCAGG
Target 5hmC	CCAGGTCCCACAGATCTATCACC^{5hm}CGGGGCTCTTCAAACTCTGCAGG
Complementary sequences	CCTGCAGAGTTTGAAGAGCCCCGGGTGATAGATCTGTGGGACCTGG

Note: Target C、Target 5mC and Target 5hmC are synthetic DNA according to musculus strain C57BL/6J chromosome 5. The red bases are the detected sites in the sequences. The probe B is modified with double biotin

group at its 5′-terminus. The probe A is modified with phosphate group at 5′-terminus. Forward primer and reverse primer are used for the PCR reaction.

2. Comparison of the proposed method with other assays for 5hmC detection

Table S2. Comparison of the proposed method with other assays

Detection strategy	Analytical range	LODs	Bisulfite	The time of assay	Sample	Refs
Fluorescence	100 fM-10 nM	34.8 fM	yes	20 h	yes	28
Fluorescence	350 aM-350pM	-	yes	24 h	yes	29
Electrochemistry	10 fM-1000pM	9.06 fM	yes	50 h	yes	30
Photoelectrochemistry	500 pM-100 nM	0.16 nM	no	12.5 h	no	32
Electrochemistry	10 pM-50 nM	1.43 pM	no	19 h	no	33
Electrochemistry	500 pM-90 nM	0.14 nM	no	12h	no	34
Fluorescence	200 aM-500 fM	-	no	8h	yes	This work

3. Oxidation process by peroxotungstate

Dinuclear peroxotungstate ($K_2[\{W(=O)(O_2)_2(H_2O)\}_2(\mu\text{-}O)] \cdot 2H_2O$) are prepared according to the reported reference.[1] The 2 μM double strand DNAs (dsDNA) or 1 μg mouse brain genomic DNA is added to the mixture containing 5 mM peroxotungstate, 1.4 M urea, 100 mM NaCl and 50 mM phosphate buffer solution (pH = 7.0). The oxidation reaction is performed under the thermal cycle condition: 10 cycles of 90 °C, 1 min and 60 °C, 10 min. The product is precipitated by ethanol and dissolved with 10 μL of distilled H_2O.

4. Standard Protocols of the Platform for 5hmC Analysis

4.1 Prepare single microbeads.

2 μL slurry of streptavidin-functionalized single microbeads (Stv-SMBs)[2] is pipetted into 200 μL centrifuge tube. Stv-SMBs are washed three times with 200 μL binging buffer (100 mM Tris-HCl, 2.5 mM EDTA, 600 mM NaCl, pH 8.0) and then suspended in the 150 μL binding buffer. A single microbead are prepared according to reported procedures.[2] Finally, a Stv-SMB with diameter of about eighty-micron (80±5 μm) and 1μL binding buffer is obtained in the 200 μL centrifuge tube. The bright-field image of Stv-SMB is shown in Fig. S6.

4.2 Conjugation of the ligation product and probe B on the microbeads.

The ligation reaction mixture (total volume 9 μL) consists of 2 nM probe A, 2 nM probe

B, 1 U Ampligase (Thermostable DNA ligase), 1×Ampligase buffer (20 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD and 0.1% Triton X-100, pH 8.3). The appropriate amounts of oxidized synthetic dsDNAs or genomic DNA samples are added to the mixture bringing to the final 10 μL volume. The reaction mixture is heated at 85 °C for 3 min to denature dsDNA and then incubated at 56 °C for 20 min to ligate probe A and probe B relying on dsDNA-5hmC. Subsequently, taking 5 μL of ligation mixture to 200 μL centrifuge tube containing the Stv-SMB to enrich the ligation products on the bead surface under vigorous shaking at room temperature for 40 min. Secondly, the Stv-SMB is incubated with 1 μL probe B (1 μM) under vigorous shaking at room temperature for 15 min to bind the probe B to the rest Stv site of the bead surface. Then the Stv-SMB is magnetically separated from mixture to remove the non-ligated probe A and excess unbound probe B. The Stv-SMB washed three times with 50 μL washing buffer 1 (20 mM Tris-HCl, 1% Tween-20, pH 8.0). Finally, the supernatant is removed, leaving only Stv-SMB in the 200 μL PCR tube.

4.3 Amplification reaction on single-microbead platform

Each 5 μL PCR reaction mixture contains 0.5 U JumpStart™ Taq DNA polymerase, 1×reaction buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin), 200 nM forward primers (FP), 200 nM reverse primers (RP), 5% formamide and dNTPs containing biotin-dATP (8 μM), dATP (8 μM), dTTP (16 μM), dCTP (16 μM), dGTP (16 μM). The 5 μL PCR reaction mixture is added to 200 μL centrifuge tube of Stv-SMB and immediately transferred into the 96 well plates of PCR system. PCR reaction is carried out in the PCR System (Applied Biosystems, USA). The cycle condition is set as: 90 °C for 30 s, 2 cycles of 90 °C for 5 s and 60 °C for 30 s, and 27 cycles of 85 °C for 5 s and 60 °C for 30 s.

After the PCR reaction, the single microbead is washed immediately three times with 50 μL of washing buffer 2 (20 mM Tris-HCl, 100 mM NaCl, pH=8) and followed by the addition of 4 μL excess Alexa Fluor 546-Stv (50 μg/mL) to stain the biotin-dATP incorporated in the PCR products on the microbead surface. After a further cycle of washing and isolation steps, the single microbead is subjected to fluorescence imaging on a confocal laser scanning fluorescence microscope.

5. Effect of PCR cycle number on this method

In this method, reaction time (expressed by the number of PCR cycle numbers) plays a crucial role in the success of the experiment. As shown in Fig. S1, when the PCR carry out 26 cycles, and the integrated fluorescence of 500 aM dsDNA-5hmC is same as blank suggesting 500 aM dsDNA-5hmC or less could not be detected and the reaction time is too short. When the PCR carry out 27 cycles, integrated fluorescence of 200 aM target dsDNA-5hmC can be clearly distinguished from the blank signal. In addition, each concentration can be clearly distinguished. When PCR carry out 28 cycles, integrated fluorescence of Stv-SMB enhanced obviously at each concentration, 200 aM dsDNA-5hmC and blank cannot be clearly distinguished. Therefore, 27 PCR cycles are selected as the optimal reaction time for this study.

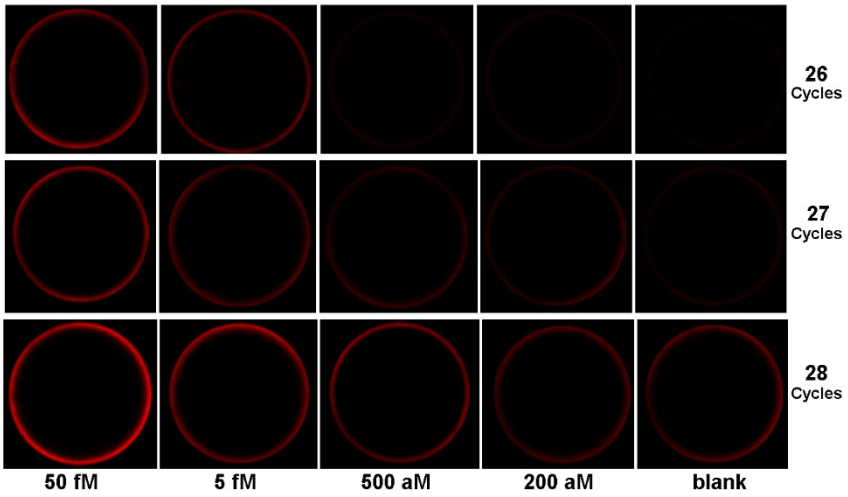

Fig. S1 The integrated fluorescence intensities of individual Stv-SMB, from left to right, dsDNA-5hmC concentration is 50 fM, 5 fM, 500 aM, 200 aM, and blank (0 aM), respectively, at different PCR cycles. Other experimental conditions are the same as described in the experimental procedure except the PCR cycle number.

6. Optimization of the concentrations of probe B

After the ligation products are bound to Stv-SMB, a large number of probes B are also bound to the Stv-SMB. Probe B can capture the PCR amplification product in solution, and initiate PCR amplification in Stv-SMB which could improve the efficiency of amplification on the microbead, Hence, the concentration of the probe B used in standard protocol 4.2 is a critical factor for POM-TPAS, and probe B is optimized with the different concentrations in the range of 1 nM to 1 μM. As depicted in Fig. S2, when the concentration of probe B is lower than 10 nM, the integrated fluorescence produced by 50 fM of dsDNA-5hmC could not be clearly distinguished from 50 fM of dsDNA-C, dsDNA-5mC and blank. The reason might be that low

concentrations of probe B would lead to low efficiency of amplification, particularly at the low levels of the dsDNA-5hmC. With increasing the concentration of probe B to 100 nM, 50 fM of dsDNA-5hmC could be clearly distinguished from dsDNA-C, dsDNA-5mC and blank. However, when the probe B dosage is further increased from 100 nM to 1 μM, one can see from Fig. S2 that the difference of integrated fluorescence value between 50 fM of dsDNA-5hmC and dsDNA-5mC is significantly reduced. This indicates that too excessive probes B may cause much target-independent nonspecific capture. Therefore, 100 nM of probe B is employed in standard protocol 4.2.

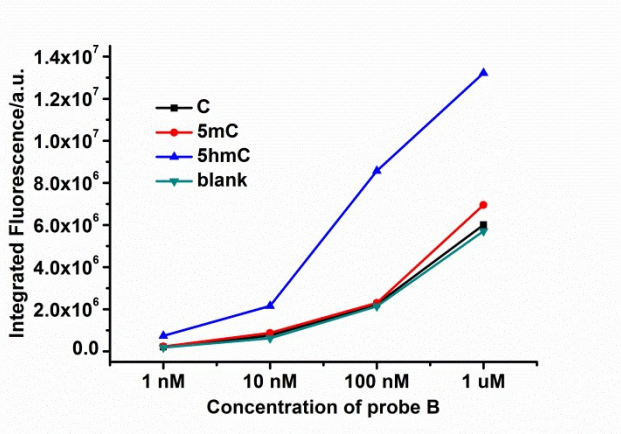

Fig. S2 The integrated fluorescence intensities of individual microbeads treated with 50 fM of dsDNA-C, dsDNA-5mC, and dsDNA-5hmC, and blank (0 fM target), respectively, under the varying concentrations of Probe B. The concentrations of Probe B, from left to right, are 1 nM, 10 nM, 100 nM, and 1 μM. Other experimental conditions are the same as described in the experimental procedure, except amount of probe B.

7. Optimization of the temperature of the ligation reaction

The ligation temperature on the proposed assay is vital for the ligation efficiency, so the ligation temperature is further studied and optimized to make sure that dsDNA-5hmC has high ligation efficiency and could be distinguished to the greatest extent from dsDNA-C and dsDNA-5mC.

As demonstrated in Fig. S3, when the ligation reaction is increased from 52 °C to 54 °C, the integrated fluorescence of dsDNA-C and blank drops sharply but the integrated fluorescence of dsDNA-5mC has no significantly change due to the Tm value of the dsDNA-5mC hybridized with probes is higher than that of dsDNA-C with probes. Fortunately, when the ligation reaction

is conducted at 56 °C, integrated fluorescence of dsDNA-5hmC has no change but the integrated fluorescence of dsDNA-5mC reduces. 50 fM of dsDNA-5hmC could be distinguished from dsDNA-C, dsDNA-5mC and blank by the maximum. While with a higher temperature of 58 °C, the integrated fluorescence of dsDNA-5hmC drops sharply owing to the low ligation efficiency at such a high temperature. Therefore, 56 °C is selected as the optimal temperature for ligation reaction in this study.

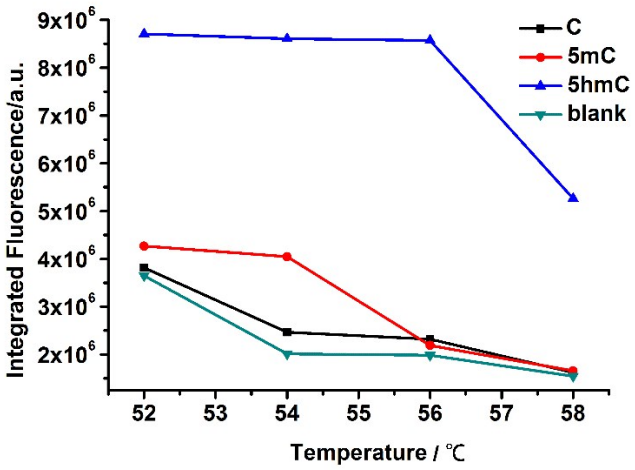

Fig. S3 The integrated fluorescence intensities of individual microbeads treated with 50 fM of dsDNA-C, dsDNA-5mC, and dsDNA-5hmC, and blank (0 fM target), respectively, under the different temperatures. The temperatures, from left to right, are 52 ℃, 54 ℃, 56 ℃, and 58 ℃. Other experimental conditions are the same as described in the experimental procedure, except temperatures.

8. Optimization of the binding time of the ligation produces and Stv-SMB

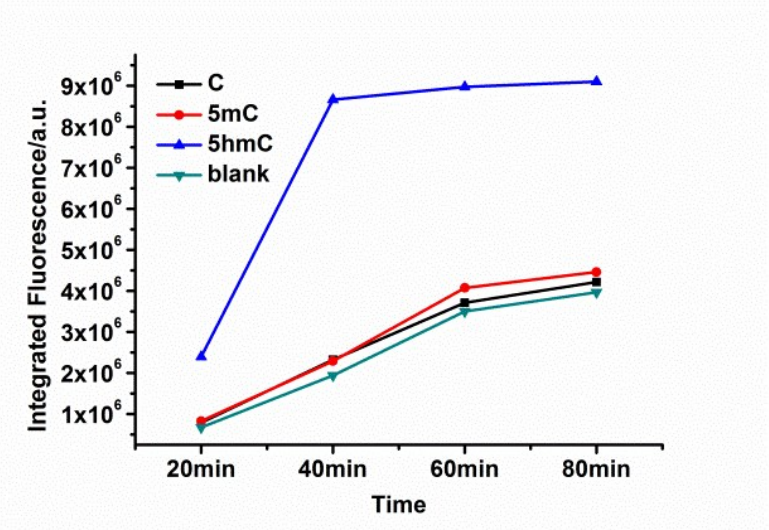

Fig. S4 The integrated fluorescence intensities of individual microbeads treated with 50 fM of dsDNA-C, dsDNA-5mC, and dsDNA-5hmC, and blank (0 fM target), respectively, under the different binding time. The binding times, from left to right, are 20 min,40 min, 60 min, and 80 min. Other experimental conditions are the same as described in the experimental procedure, except binding time.

According to the principle, the amplification efficiency will be seriously influenced by the amount of ligation products on single microbead. To ensure that the ligated products can be captured completely on Stv-SMB, the effect of the binding time of ligation produces and microbead is investigated in this study. The integrated fluorescence is investigated at 20 min, 40 min, 60 min and 80 min of binding time, respectively. It can be seen from Fig. S4 that as the binding time increases from 20 min to 60 min, the difference of the integrated fluorescence between dsDNA-5hmC and dsDNA-C/ dsDNA-5mC/blank is sharply increased and then decreases. When the binding time is conducted at 40 min, this difference reaches the maximum. When the binding time is 80 min, the distinction between dsDNA-5hmC and dsDNA-C/ dsDNA-5mC/blank is essentially unchanged. In consideration of all of these parameters, 40 min binding time is selected to be the optimum.

9. Quantitative detection of dsDNA-5hmC in mouse brain genomic DNA

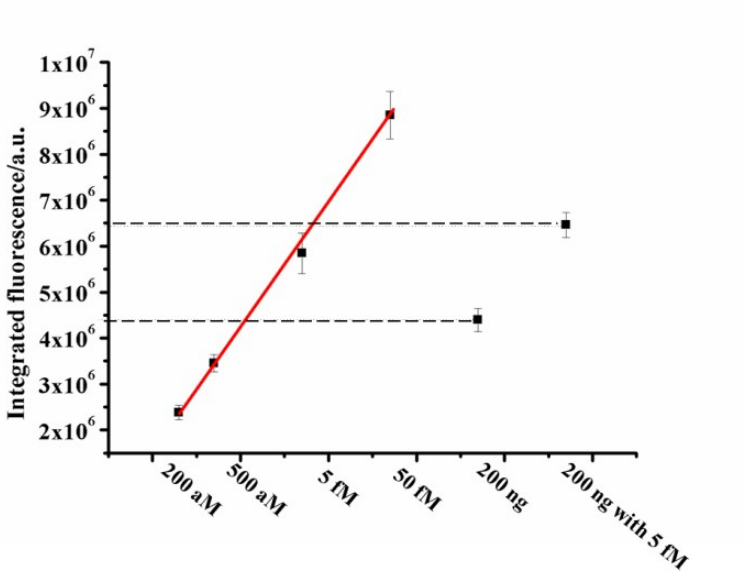

Fig. S5 The simultaneously constructed calibration curve for quantitative detection of dsDNA-5hmC in mouse brain genomic DNA.

10. The bright-field image of streptavidin-functionalized single microbead (Stv-SMB)

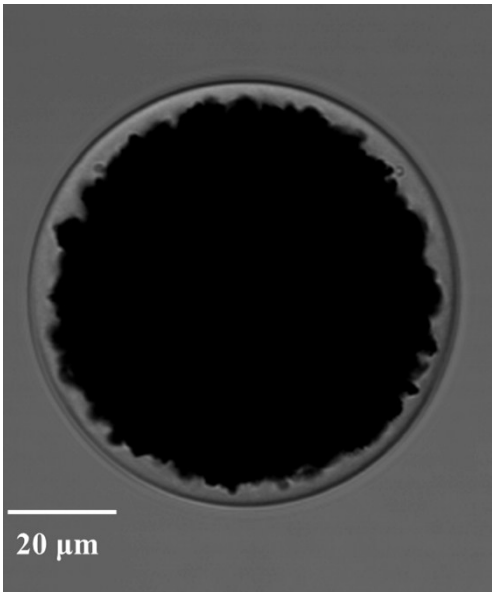

Fig. S6 The bright-field image of streptavidin-functionalized single microbead (Stv-SMB)

1. G. Hayashi, K. Koyama, H. Shiota, A. Kamio, T. Umeda, G. Nagae, H. Aburatani and A. Okamoto, *J. Am. Chem. Soc.*, 2016, **138**, 14178-14181.

2. X. Zhang, C. Liu, L. Sun, X. Duan and Z. Li, *Chem. Sci.*, 2015, **6**, 6213-6218.