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Technical Note

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An Integrated Approach Based on a DNA Self-Assembly Technique for Characterization of Crosstalk among Combinatorial Histone Modifications

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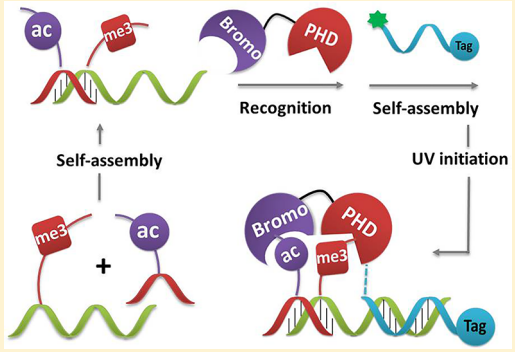
Supporting Information

ABSTRACT: Combinatorial histone post-translational modifications (HPTMs) form a complex epigenetic code that can be decoded by specific binding proteins, termed as readers. Their specific interplays have been thought to determine gene expression and downstream biological functions. However, it is still a big challenge to analyze such interactions due to various limitations including rather weak, transient, and complicated interactions between HPTMs and readers, the high dynamic property of HPTMs, and the low abundance of reader proteins. Here we sought to take advantage of DNA-templated and photo-cross-linking techniques to design a group of combinatorial histone PTM peptide probes for the identification of multivalent interactions among histone PTMs and readers. By use of trimethylation on histone H3K4 (H3K4me3) and phosphorylation on H3T3, we demonstrated that this approach can be successfully utilized for identification of the PTM crosstalk on the same histone. By use of H3K4me3 and acetylation on H4K16, we showed the potential application of the probe in the multivalent interactions among PTMs on different histones. Thus, this new chemical proteomics tool combined with mass spectrometry holds a promising potential in profiling of the readers of combinatorial HPTMs and characterization of crosstalk among multiple PTMs on histones and can be adapted for broad biomedical applications.



In eukaryotic cells, histones (H2A, H2B, H3, and H4), the fundamental proteins wrapped by genomic DNA in the nucleosome, are dynamically decorated by a large number and variety of post-translational modifications (PTMs) such as lysine acetylation, methylation, and ubiquitination.[1−4] Histone post-translational modifications (HPTMs) are thought to be a group of epigenetic marks that have been linked to gene transcription, DNA damage, and DNA replication.[5] Modifications on histones not only directly alter chromatin structure but also serve as signal platforms to recruit specific binding partners, termed as "readers". These histone readers as well as their complexes fundamentally determine the functional outcome of histone PTMs and downstream biological functions.[6] Thus, the dysregulation of this recognition and translation process further causes the occurrence of many human diseases, such as cancer.[7,8] Emerging evidence suggests that the high density of histone marks may raise PTM-mediated crosstalk,[9] which will promote or repel the recruitment of reader proteins, further altering transcription. The readout of one histone PTM may be regulated by another adjacent PTM;[2,6] for example, the phosphorylation of serine 10 on histone H3 (H3S10ph) promotes acetylase Gcn5 to acetylate histone H3 at lysine 14 during gene activation. However, this H3S10ph can repel the binding of heterochromatin protein 1 (HP1) with its adjacent methylated H3K9 during mitosis. This crosstalk between methylation and phosphorylation modulates the binding of HP1 with histone H3 and thus alters chromosome alignment and segregation.[10] In addition to crosstalk on *cis*-histone, where two PTMs occur on the same histone protein, interplays of PTMs can span more than one histone (*trans*-histones); for example, trimethylation of lysine 4 on histone H3 (H3K4me3) and acetylation of lysine 16 on histone H4 (H4K16ac) can constitute a *trans*-histone PTM pattern to simultaneously interact with the second PHD finger and neighboring bromodomain of human BPTF.[11] The interaction forming between combinatorial PTMs and readers are very complicated, and their identification and characterization is essential for understanding the mechanism and function of histone PTMs.

Although a number of combinatorial PTMs have been identified on histone tails,[3] their effects on the PTM−reader interaction have not yet been carefully examined, preventing us from understanding the crosstalk among histone PTMs. To date, the main approaches to identify the interaction between

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histone PTMs and readers is to mimic PTM-based affinity purification from synthetic histone peptides to a reconstituted nucleosome.[2] However, due to various limitations including rather weak, transient, and complicated interactions between HPTMs and readers, the high dynamic property of HPTMs, and the low abundance of reader proteins,[12] it is still a big challenge to analyze the multivalent interactions among histone PTMs and readers. A sensitive and applicable approach is expected to address the issues.

Recent reports showed that peptide probes carrying photo-cross-linking groups can improve the capture of the histone reader by converting weak noncovalent interactions into irreversible covalent bonds under the UV initiation.[13,14] In addition, DNA-templated technology allows the desired chemical groups to be delivered to the specific site via the self-assembly of complementary double-stranded DNA for detection of proteins[15−17] as well as histone readers.[18]

Here we sought to take advantage of the photo-cross-linking technique and DNA-templated chemistry to design a mimic histone peptide probe for the identification of multivalent interactions among histone PTMs and readers. By use of H3K4me3 and H3T3ph, we demonstrated that this probe can be successfully utilized for identification of the crosstalk on *cis*-histone. By use of H3K4me3 and H4K16ac, we showed the potential application of the probe in the multivalent interactions among PTMs and readers on *trans*-histone. Thus, a new chemical proteomics tool for profiling the crosstalk among HPTMs and readers was successfully developed.

EXPERIMENTAL SECTION

Materials and Instruments. Succinimidyl-ester diazirine (SDA) and streptavidin agarose beads were from Thermo Fisher Scientific Ltd. Bovine serum albumin (BSA) was purchased from Sigma−Aldrich. All modified oligonucleotides were from Sangon Biotech (Shanghai) Co., Ltd. Modified peptides were synthesized by Beijing SciLight Biotechnology Ltd. Co. Protein identification was performed on an EASY-nLC 1000 Nano-HPLC-Orbitrap Q-Exactive Mass Spectrometer (Thermo Fisher Scientific). Oligonucleotides were quantified by a NanoDrop 2000C (Thermo Fisher Scientific). All gel images were captured by a Bio-Rad gel imager and fluorescence imaging with 365 nm excitation.

Fluorescence Labeling Experiment. Characterization of synthetic peptides and oligonucleotides is shown in Figures S2 and S3. Design and preparation of the probes is described in the Supporting Information. The binding probe and PHD_{BPTF} were incubated in binding buffer (50 mM Tris-HCl, pH = 7.8, 200 mM NaCl, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM $ZnCl_2$, 2 mM DTT, 0.005% NP-40) at 4 °C overnight. The fluorescein labeled capture probe was added to the mixture and kept at room temperature for another 1 h to perform DNA hybridization. After irradiation for 6 min on ice using a UVP CL-1000 at 365 nm, the sample was concentrated to dry powder and redissolved by loading buffer. The protein sample was boiled for 10 min at 95 °C and resolved by 12% SDS-PAGE and further analyzed by scanning the gels using a Bio-Rad gel imager.

Affinity Pull-Down Experiment. Similarly, the binding probe was incubated with PHD_{BPTF} at 4 °C overnight first and then with biotin labeled capture probe at room temperature for 1 h. The sample was irradiated for 6 min on ice and transferred into prewashed streptavidin agarose beads at 4 °C for 4 h. The beads were washed with wash buffer 1 (2 M NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.4, 0.01% Triton X100) two times and wash buffer 2 (2 M NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.4, 0.1% Triton X100) one time to remove the nonspecific protein. Loading buffer was added to the resins, and the sample was heated to 95 °C for 10 min. Finally, the mixture was centrifuged at 500*g* for 1 min, and the supernatant was loaded onto the gels to analyze by SDS-PAGE and LC/MS/MS.

Mass Spectrometry Analysis of Protein Samples. The proteins were resolved on SDS-PAGE and visualized by silver staining, and then, the corresponding bands were subjected to in-gel digestion. The resulting peptides were loaded into a Nano-LC system equipped with a C18 column and electrosprayed directly into an Orbitrap Q-Exactive mass spectrometer. The resulting MS raw data were searched using MASCOT. For the detailed parameters of LC/MS/MS and data searching, please see the Supporting Information.

Identification of the Crosstalk of PTMs Existing on Different Histones. Binding probe 1 and binding probe 2 (12 μM) were premixed at room temperature for 2 h to assemble the two PTMs containing the multivalent binding probe. Then, the protein was added (6 μM) and incubated with the binding probe overnight at 4 °C. Subsequently, the fluorescein labeled capture probe (12 μM) is added to the mixture at room temperature for 2 h to finish the second assembly. After the sample was exposed to UV at 365 nm on ice for about 6 min to trap the target covalently, it was concentrated to dry powder and dissolved by moderate loading buffer. The sample was heated at 95 °C for 10 min and loaded into gels and analyzed by 12% SDS-PAGE and further imaged by a Bio-Rad gel imager.

RESULTS AND DISCUSSION

Analysis Strategy and Preparation of HPTM Probes. Combinatorial histone PTM states may occur in several possible situations spatially, including one in which the PTMs coexist on the same histone molecule (*cis*-histone) or on different histone proteins (*trans*-histone). In this study, we developed the HPTM probes for identification of the crosstalk in the two different patterns. As a sensitive subunit to recognize histone PTMs, BPTF has two different domains to be recruited cooperatively by histone H4K16ac and H3K4me3, and still, this protein's binding with H3K4me3 is disrupted by neighboring threonine 3 phosphorylation (H3T3ph),[19] so, in this study, we use BPTF as a primary example.

A brief description of the strategy to identify the PTM crosstalk on *cis*-histone is shown in Figure 1. Two DNA probes are designed, prepared, and characterized (Figures S1 and S4), named as the binding probe and capture probe, respectively. The binding probe includes a mimic peptide with the desirable histone PTMs and amino acid sequence, as well as a segment of DNA sequence. While the capture probe is composed of a complementary DNA sequence and a photo-cross-linking group, as well as a fluorescence or biotin tag. Briefly, binding probes carrying single HPTM or combinatorial HPTMs were used for incubation with the binding proteins, respectively. Second, the capture probe is added, and thus, the cross-linking groups are brought to the adjacent position of the binding protein via self-assembly of two complementary single-stranded DNA molecules. Third, a covalent bond is further formed between the capture probe and its target protein under the 365 nm photoinitiation. Fourth, the binding protein can be detected by in-gel imaging or affinity enrichment with the corresponding tag at the tail end of capture probe. Finally, according to the

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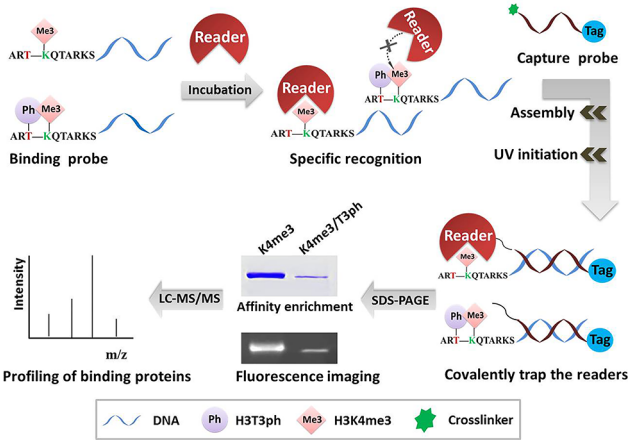

Figure 1. Schematic representation for analysis of crosstalk among HPTMs on *cis*-histone using DNA-templated HPTM probes. H3K4me3 binding probes with or without H3T3ph are incubated with reader proteins and assembled with a capture probe, and then, appropriate UV photoinitiation enables a covalent bond to form between reader protein and probe. Captured protein is further analyzed by in-gel imaging or affinity enrichment followed by mass spectrometry analysis.

outcome of two probes, we can determine the multivalent interactions between HPTMs and readers, further revealing the crosstalk among PTMs on histones.

Feasibility of the Approach for Characterization of the Interaction between Combinatorial HPTMs and Readers. Previous studies have shown that H3T3ph has a profound impact on the binding of PHD domain with H3K4me3;[19] thus, we expressed the PHD domain of BPTF as the reading target and prepared the probes of H3K4me3 and H3K4me3T3ph as the combinatorial HPTM pattern. A group of parallel *in vitro* experiments were designed to examine the feasibility of the approach to sense the PHD_{BPTF} binding mediated by HPTM crosstalk. The binding probe with H3K4me3 was first incubated with the PHD_{BPTF} domain and subsequently assembled with a capture probe and then followed by a photo-cross-linking reaction. After SDS-PAGE separation, we observed that a distinct fluorescent band appeared at the correct position on the gel (Figure 2, lane 1), while the same experiment from the probe with H3K4me3 and H3T3ph got a very weak band (Figure 2, lane 2), which means that H3T3ph repelled the interaction between H3K4me3 and PHD_{BPTF} as reported.[19] This result demonstrated that the pair of probes is sensitive enough to detect the effect of phosphorylation on the HPTM−reader interaction and to determine the crosstalk between HPTMs. In addition, no detectable protein labeling was observed without UV irradiation (Figure 2, lanes 3 and 4), which suggested that the covalent bonding of probe indeed improved the capture of readers. Compared to the input control, it can be observed that the molecular weight of linked PHD_{BPTF} was increased due to addition of the cross-linked probe to the target. From comparative analysis with and without UV initiation, it can be seen that the photo-cross-linker can be delivered to proper position and that it covalently captured the reader proteins.

Analysis of HPTM Crosstalk in a Complex Environment. We next sought to analyze whether the approach can be used for HPTM crosstalk in a complicated environment. Thus, we examined selectivity of the pair of probes to PHD_{BPTF} in BSA and whole cell lysates background, respectively. Figure 3

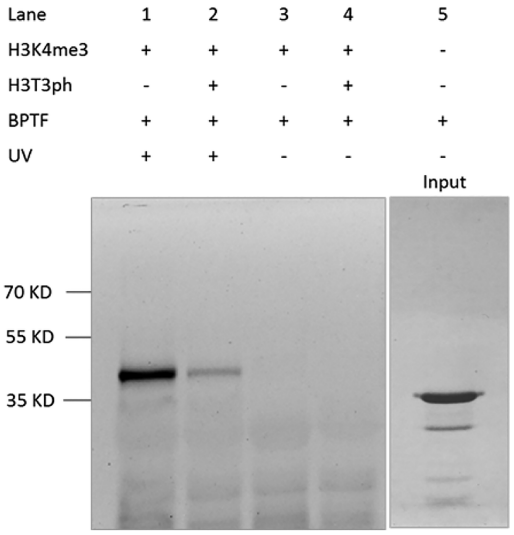

Figure 2. Detection of the different binding of PHD_{BPTF} to combinatorial HPTMs using a DNA-templated peptide probe via fluorescence imaging. PHD_{BPTF}: 6 μM (6 μg), probe: 12 μM. Lanes 1 and 2: labeling experiment performed by probes H3K4me3 and H3K4me3T3ph. Lanes 3 and 4: the same as lanes 1 and 2 but without UV irradiation. Lane 5: input control of protein sample (PHD_{BPTF}: 6 μg) detected by Coomassie blue staining.

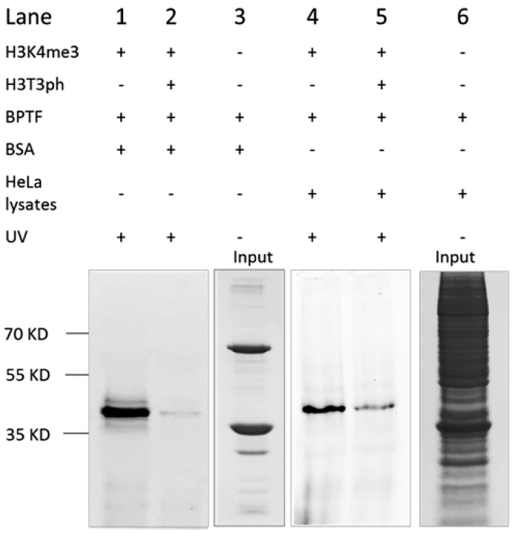

Figure 3. Detection of different PHD_{BPTF} binding to H3K4me3 in the presence of BSA (lanes 1 and 2) and the whole cell lysate (lanes 4 and 5) by fluorescence imaging. Lanes 3 and 6: input protein samples contaminated by BSA (BSA = PHD_{BPTF} = 6 μg) and HeLa lysates (HeLa lysates/PHD_{BPTF} = 30:6 μg) detected by Coomassie blue staining.

showed the differential bands from two probes in two backgrounds, which are consistent with those in a standard sample. Compared to the input control of the samples contaminated by BSA and HeLa lysates, the approach showed specific labeling to target protein. It demonstrated that the probes can specifically capture the target protein in a complicated environment, further revealing that the approach could be used in complex sample mixtures to measure the different interactions between combinatorial HPTMs and readers.

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Reader Enrichment Regulated by Combinatorial HPTM Probe. To test whether the combinatorial HPTM probe could be used to enrich the target domain, we further performed a group of pull-down experiments in a pure and complicated background using a biotin and streptavidin isolation system. Compared to the input control (samples before enrichment, lane 3 in Figure 4 for BSA spiked-in and lane 6 for HeLa spiked-in experiment), six pull-down experiments showed that the H3K4me3 probe achieved the specific enrichment to the protein, matching the expected molecular weight of PHD_{BPTF} (lanes 1, 4, and 7 in Figure 4), and further MS analysis confirmed this (Figure S7 and Table S1). Obviously, the product enriched by the probe with H3T3ph significantly decreased (lanes 2, 5, and 8), compared to those from probe H3K4me3. These results demonstrated that the functionalized HPTM probe possesses excellent specific enrichment to its target even in a complex environment and further is allowed to combine with MS for screening the binding proteins of combinatorial HPTMs. In a traditional photolinked peptide probe, photolinked groups are usually localized at a PTM adjacent amino acid to obtain specific linking to binding proteins. However, it may bring the spatial hindering for the interaction among multiple neighboring PTMs with binding proteins. In our approach, the PTMs and photolinked groups are localized in two probes. After the probe with multiple PTMs traps the binding protein, the photoreactive group is brought close to the binding protein via DNA hybridization and further linked to the target via UV light initiation. This proposed approach eliminates the problems of internal localization of photolinked groups affecting multiple neighboring HPTM−reader recognition and thus is more favorable for characterization of crosstalk among combinatorial HPTMs.

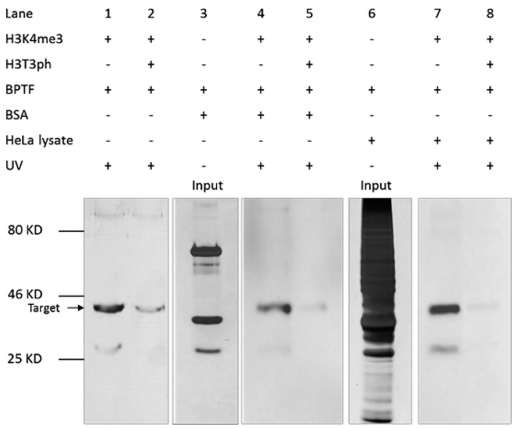

Figure 4. Detection of the products enriched by the probe carrying H3K4me3 with or without H3T3ph in different background by silver staining. The recombinant PHD_{BPTF} (lanes 1 and 2, 12 μg) and recombinant PHD_{BPTF} contaminated by BSA (lanes 4 and 5, BSA = PHD_{BPTF} = 12 μg) and whole cell lysates (lanes 7 and 8, HeLa lysates/PHD_{BPTF} = 60/12 μg). Lanes 3 and 6: BSA and HeLa lysate contaminated samples before the enrichment (50% input).

Characterization of Interaction between Combinatorial PTMs on *trans*-Histone. It has been discovered that the crosstalk between HPTMs can occur on different histone proteins. A typical example is the synergistic interaction of the second PHD finger and neighboring bromodomain of human BPTF with H3K4me3 and H4K16ac.[11] Although it has been thought that such crosstalk plays an important role in chromosome remodeling and gene transcription, these kinds of multivalent interactions between combinatorial HPTM patterns and readers remain poorly understood due to the lack of a proper analytical method. In addition to the issues in *cis*-histone, a big challenge is how to install the spatial layout of two PTMs on *trans*-histones. According to the distance and spatial position between H3K4me3 and H4K16ac, a 20 base pair rigid DNA duplex with a histone peptide can bind with PHD and the bromodomain simultaneously.[20] Based on this knowledge, we attempted to explore the identification of the above multivalent interactions on *trans*-histone using a DNA-templated probe. A brief description of the strategy is shown in Figure 5a. We first prepare two partially complementary binding probes that contain H3K4me3 and H4K16ac, respectively. Second, these two binding probes are coupled together via the self-assembly of complementary double-

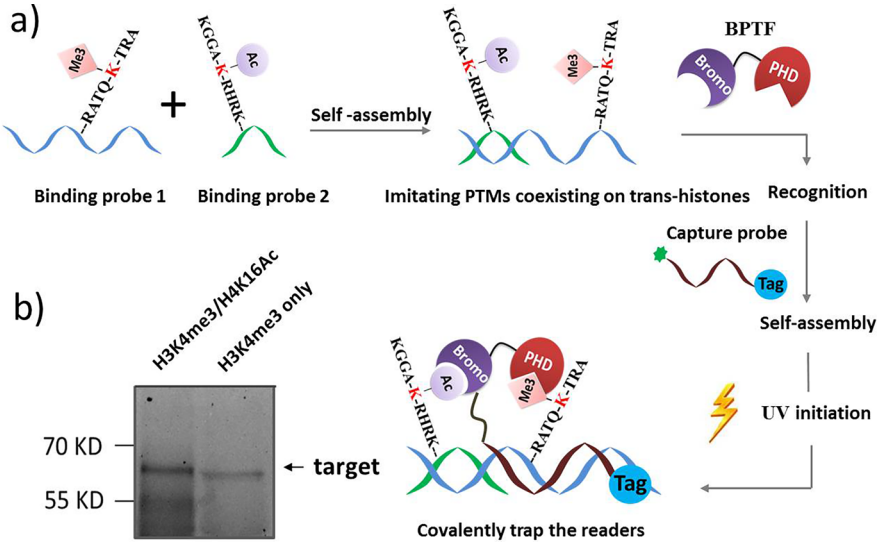

Figure 5. (a) Schematic representation for analysis of crosstalk among HPTMs on *trans*-histones using DNA-templated HPTM probes. (b) Fluorescence imaging of BPTF using a DNA-templated multiple peptide probe. Arrows indicate the labeled tandem PHD-$Bromodomain_{BPTF}$.

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stranded DNA. Third, the dual HPTM probe imitating *trans*-histone is further incubated with dual domains. Fourth, the addition of a capture probe would initiate binding probe/capture probe hybridization that brings the cross-linker close enough to the targets. Fifth, UV light initiates the photo-cross-linking reaction. Finally, the captured proteins can be detected by a fluorescent tag. Using this approach, we successfully captured the tandem PHD-bromodomain, as shown in in-gel imaging (Figure 5b). An enhancement of the labeling of the target protein was achieved by using the dual-PTM probe as opposed to the single-PTM probe, suggesting that the methods can detect the simultaneous binding of readers by PTMs existing on two histones.

CONCLUSION

In summary, we have developed a combined approach based on a peptide−DNA assembly technique for the detection and identification of the multivalent interaction between combinatorial histone PTMs and readers. Our results showed that the group of functional probes can selectively and sensitively sense the effect of one PTM to its nearby PTM upon binding of the recognition domain, even in a complicated environment. We further validated that this approach can be extended to characterize multivalent interactions among readers and PTMs on two different histones by designing a double-stranded DNA as a medium to imitate the two PTM states. This probe is not restricted to known histone binding proteins and therefore permits an unbiased survey of interactions between histone binding modules and a multitude of histone modification states, suggesting that this novel approach combined with mass spectrometry is a potential tool for characterization of crosstalk among combinatorial HPTMs, which acts as an issue in the epigenetic field. Future studies will be readily expanded to discover complicated interactions between HPTMs and readers. This work also suggests that the integrated technique of DNA-templated photo-cross-linking enables us to construct the functional mimic probe for broad biomedical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b05174.

Synthesis and characterization of the HPTM probe, peptides, and DNA, expression and characterization of protein, and MS validation of enriched protein (PDF)

Data for recombinant PHD, recombinant PHD-Bromo, and enriched PHD (XLSX)

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Notes

The authors declare no competing financial interest.

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