Supporting Information

# Branched immunochip-integrated pairwise barcoding amplification exploring the spatial proximity of two post-translational modifications in distinct cell subpopulations

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**Experimental Sections**

**Materials and Chemicals.** Dibenzocyclooctyne-sulfo-N-hydroxysuccinimidyl ester (DBCO-Sulfo-NHS ester), 3-mercaptopropyl trimethoxysilane) trimethoxysilane (MPTS), n-γ-maleimidobutyryl-oxysuccinimide ester (GMBS), streptavidin and collagen A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-H3K4me1, H3K4me3, H3K27me3 and H3K27ac antibodies were from Cell Signaling Technology (Massachusetts, USA). Mouse anti-H3K4me3 antibody was from Sigma-Aldrich. Other antibodies with biotin were from Thermo Fisher Scientific. All oligonucleotides used in this work were provided by Sangon Biological Co. Ltd (Shanghai, China), and listed in Table S1. Amicon Ultra-0.5 NMWL 100-kDa centrifugal filter and Amicon Ultra-0.5 NMWL 30-kDa centrifugal filter were obtained from Merck Millipore. T4 DNA ligase and phi 29 DNA polymerase were purchased from New England Biolabs Ltd. (Beijing, China). Poly(dimethylsiloxane) (PDMS) was from Dow Corning (Germany). QuickBlock™ blocking buffer, QuickBlock™ Primary Antibody Dilution Buffer and QuickBlock™ Secondary antibody dilution buffer were obtained from Beyotime Biotechnology (Shanghai, China).

**Preparation of DNA-Conjugated Antibody.** Rabbit anti-Mouse IgG and Donkey anti-Rabbit IgG were first conjugated with DBCO-Sulfo-NHS following a standard procedure. In brief, 50 μg antibody diluting into 50 μl 1×PBS was mixed with 1.2μl DBCO-Sulfo-NHS ester (2 mM, dissolved in dimethylsulfoxide) and rotated for 2 h at room temperature (RT). Then the sample was concentrated to 30 μl by Amicon Ultra-0.5 NMWL 30-kDa centrifugal filter, and unreacted DBCO-Sulfo-NHS ester was removed. 6 μl azide modified-DNA probe sets (100 μM) were added into the DBCO-functionalized antibody respectively. The conjugation reaction lasted for 1 week at 4 °C using a rotator. The product was passed through Amicon Ultra-0.5 NMWL 100-kDa centrifugal filter for three times to remove the free DNA probes as much as possible. 30 μl product of DNA probe-conjugated antibody was obtained for each PTM.

**Fabrication of Microfluidic Chip.** The coverglass was treated with MPTS (4%, v/v) for 30 min and washed with ethanol for three times. After dried at 100 °C, the coverglass was treated with 0.01 μM GMBS crosslinker for 30 min at RT. Then 10 μg/mL of streptavidin in 1×PBS solution was incubated on the coverglass for 1 h. After washing again, biotinylated antibodies (20 μg/mL) were incubated for another 1 h at RT, and washed 3 times to remove excessive molecules. At last, the modified coverglass was treated with 2% BSA and 0.05% Tween 20 in 1×PBS to eliminate the nonspecific adsorption. The microfluidic chip was designed and fabricated according to our previous protocol. The mold was fabricated by photolithography using a positive photoresist (AZ 50XT) on a silicon wafer. A layer of PDMS with the ratio of oligomer and crosslinker at 10:1 (w/w) was poured onto the mold, degassed, and cured for 3 h at 70 °C. Then the cured PDMS was peeled from the mold, treated with plasma and bonded onto the antibody-modified coverglass.

**Cell Preparation and Immunocapture.** MCF-10A, MCF-7, MDA-MB-231, BT474 and A549 cells were cultured in Dulbecco's modified Eagle's medium 100 U/mL of penicillin, 100 g/mL of streptomycin and 10% fetal-bovine serum at 37 °C. PDMS chambers with 4 mm diameter were sterilized and coated with collagen A. For each adherent cell imaging sample, about 6000 cells were seeded in a PDMS chamber and incubated at 37 °C overnight. The cell suspensions were prepared using trypsin. The fixation of cells was performed using 4% formaldehyde for 10 min at RT. Then the cells were permeabilized with 0.5% Triton X-100 (diluted in 1×PBS) for 5 min at RT. For immunocapture, $1\times10^5$ cells/ml of fixed cells were flown into the chip and incubated for 10 min. After incubation, the chip was washed three times to remove the uncaptured cells. Then pairwise barcoding amplification was performed to analyze corresponding cell subpopulations. Detailed process was in the following Pairwise Barcoding Amplification section.

**Preparation of Nonadjacent Sites-Specific Circularized DNA Probes.** The circularized DNA template probes for nonadjacent sites were prepared by a series of reactions. In brief, 20 μM linker probes were hybridized with 10 μM padlock probes in 1×T4 DNA ligase buffer for 2 h at 55 °C. Then T4 DNA ligase (500 U) was added and incubated at 37 °C for 3 h for the probes ligation. At last, Exonuclease I and Exonuclease III were added and thoroughly digested the left linear DNA overnight. The circularized probes were stored at 4 °C before use.

**Pairwise Barcoding Amplification.** First, the fixed cells were incubated in QuickBlock™ blocking buffer at RT for 1 h for blocking. Primary antibodies H3K4me1, H3K4me3, H3K27me3 and H3K27ac were diluted in QuickBlock™ Primary Antibody Dilution Buffer at the ratio of 1:800, 1:400, 1:1600 and 1:100. Then the blocked cells were

incubated with specific primary antibodies at 4 °C for 12 h. After washing three times (10 min each) using 1×PBST (0.01% Tween-20 in PBS), DNA-conjugated secondary antibody (diluted in QuickBlock™ Secondary antibody dilution buffer at the ratio of 1:50) were added to the cells and reacted for 1h at RT. Three times of washing with 1×PBST were performed. Notably, the secondary antibody-conjugated DNA probes were worked to hybridize with proximity-related Linker-P and Pad-P probes (200 nM) in in 1×T4 DNA ligase buffer. Next, 10 U T4 DNA ligase were added to complete the circularization. Linear Linker-P and Pad-P probes on nonadjacent PTM sites were displaced by Disp-1 and Disp-2 probes (200 nM each) at 37 °C for 1 h. Prepared circularized DNA templates for nonadjacent sites (100 nM) were incubated with the cells, and RCA was performed with phi29 DNA polymerase (10 U) and dNTP (2 mM) for 2 h at 37 °C. 200 nM of each fluorescent DNA probes were hybridized with RCA products in 2×SSC buffer with 20% (v/v) formamide for 30 min. The nuclei were stained by DAPI. Three times of washing were done for each reaction.

**Cell Imaging and Data Analysis.** Fluorescence imaging was carried out using a laser scanning confocal microscope (TCS SP8 STED 3X, Leica) with a 20× dry objective or a 63× water objective (NA 1.2). Z-stacks of cell images were collected at intervals of 0.5 μm (20 planes in total). For each control, cells were randomly chosen. Stacks of images were overlaid, and performed colocalization analysis by LAS X.[1] The spot counts and signal intensity of cells were extracted by Image J.

**Table S1.** Sequence information for oligonucleotides used in this research.

| Name | Sequences (5′-3′) |
|---|---|
| P-PTM1 | **$N_3$**-AAAAAAAAAAAAAAAAAAAAGACGCTAATAGTTAAGACGCTT |
| L-PTM1 | GACGCTAATAGTTAAGACGCTT |
| Pad-PTM1 | **P**-CTATTAGCGTCAGTAGAGCTTACTCACAGCCAGCATCACAGCCGTCACAAGCGTCTTAA |
| Disp-PTM1 | GACGCTAATAGTTAAGACGCTTATATGACAG |
| P-PTM2 | **$N_3$**-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATATGACAGAACTAGACACTCTT |
| L-PTM2 | ATATGACAGAACTAGACACTCTT |
| Pad-PTM2 | **P**-GTTCTGTCATATAGTGATGAGCCTTCCTCGGTACGGTCTGTAGCCGCTAAGAGTGTCTA |
| Disp-PTM2 | AAGACGCTTATATGACAGAACTAGACACTCTT |
| L-proximity | **P**-GTTCTGTCATATACAAGCGTCTTAA |
| Pad-proximity | **P**-CTATTAGCGTCCAGTGAATTATACCCGGTCGCTTCTTTATGCCGTCAAGAGTGTCTA |
| FAM-probe | **FAM**-TTATACCCGGTCGCTTCTTTAT |
| Cy5-probe | **Cy5**-TGAGCCTTCCTCGGTACGGTCTGTA |
| Cy3-probe | **Cy3**-AGAGCTTACTCACAGCCAGCATCACA |
| FP-PTM1 | **Cy5**-AAGCGTCTTAACTATTAGCGTC |
| FP-PTM2 | **Cy3**-AAGAGTGTCTAGTTCTGTCATA |

[a] $N_3$ and P represent the azide and phosphate group modified at the 5’ end of the DNA probes.

[b] FAM, Cy3 and Cy5 represent the modified fluorophores with different excitation and emission wavelengths.

**Table S2.** Primary antibody information for PTMs.

| Name | Company | Item number |
|---|---|---|
| Mono-Methyl-Histone H3 (Lys4) (D1A9) XP® Rabbit mAb | Cell Signaling Technology | 5326T |
| Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb | Cell Signaling Technology | 9751T |
| Acetyl-Histone H3 (Lys27) (D5E4) XP® Rabbit mAb | Cell Signaling Technology | 8173T |
| Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb | Cell Signaling Technology | 9733T |
| Anti-Trimethyl Histone H3 (Lys4) Antibody, clone CMA304 | Sigma-Aldrich | 05-1339-S |

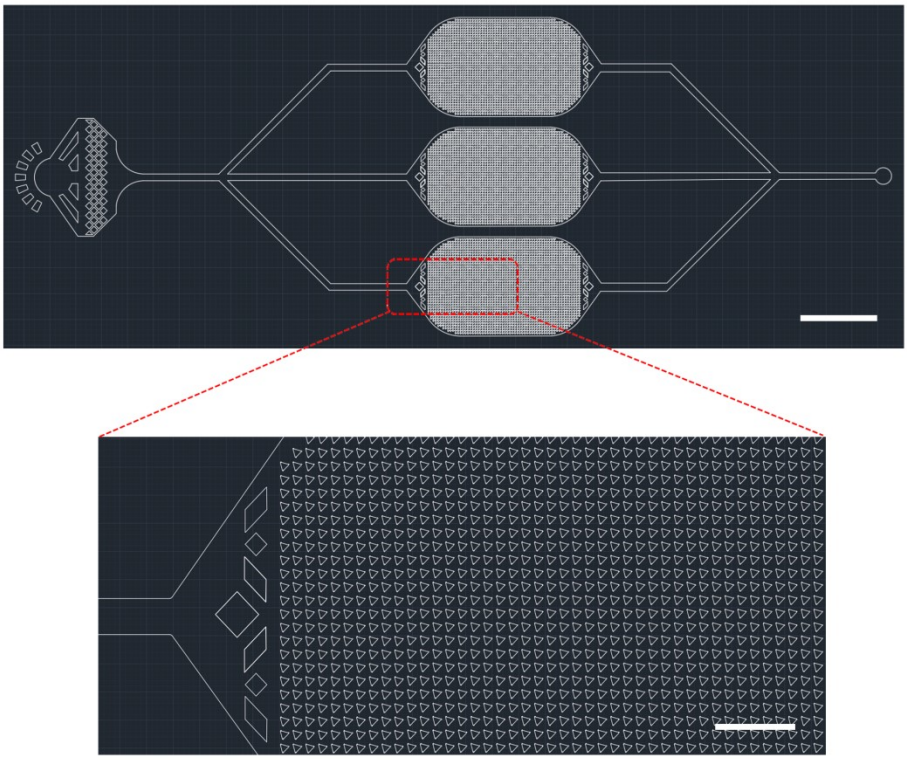

**Figure S1.** Design of the proposed microfluidic branched immunochip. Scale bar, top: 3 mm; bottom: 700 um.

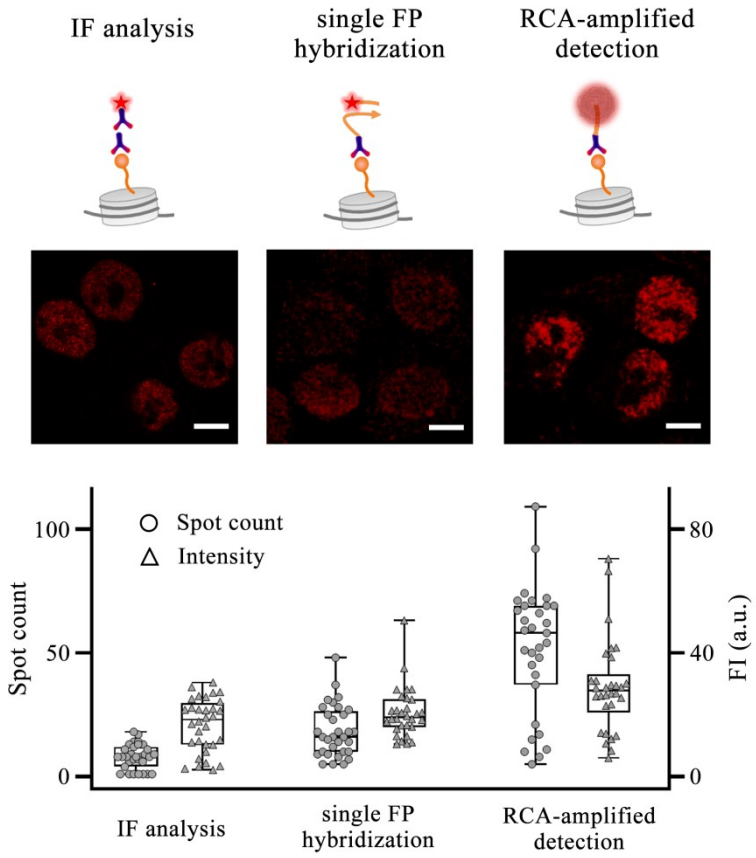


**Figure S2.** Comparison of immunofluorescence, single FP hybridization and RCA for detecting PTMs. (A) Top: cell images of H3K4me3 by different detection methods in MCF-10A cells. Scale bar, 5 μm. Bottom: corresponding statistical analysis of single-cell spot counts and fluorescence intensity for each method (N=32). N: the number of analyzed cells.

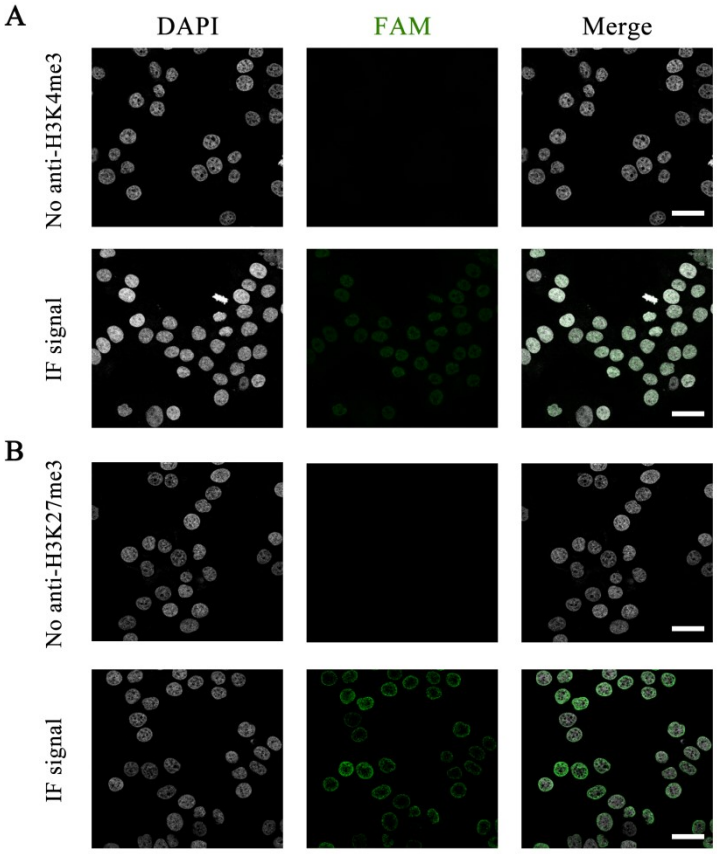


**Figure S3.** Immunofluorescence imaging of (A) H3K4me3 and (B) H3K27me3 in MCF-10A cell line. Rabbit anti-Mouse and Donkey anti-Rabbit fluorophore-labeled secondary antibodies were used. Samples with no primary antibodies were set as negative controls. Scale bar, 30 μm.

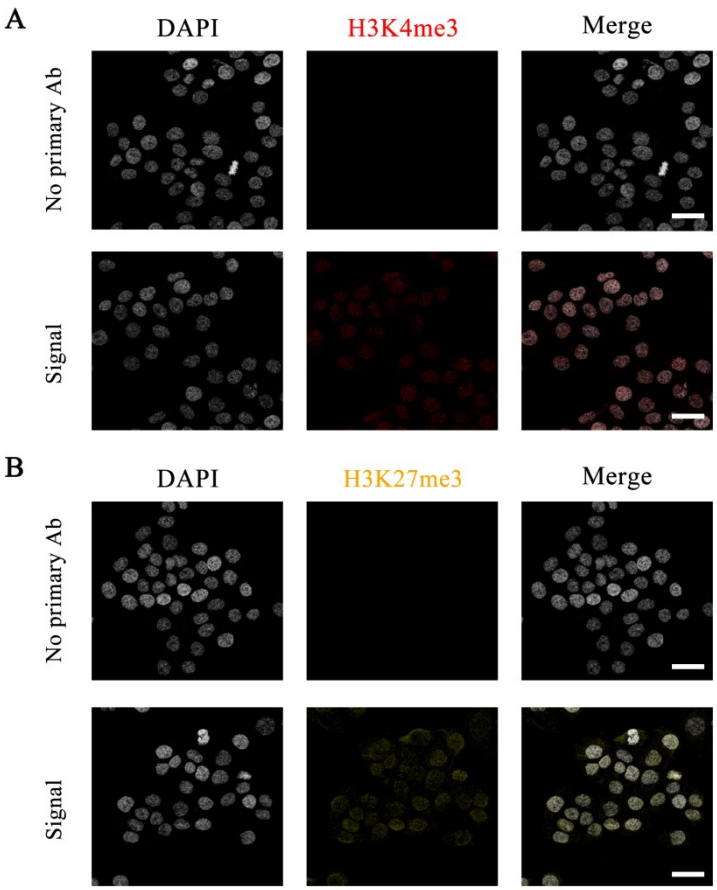


**Figure S4.** Cell images for detecting individual PTM (A) HK4me3 and (B) H3K27me3 with single FP hybridization. Scale bar, 30 μm. MCF-10A cell line was used here.

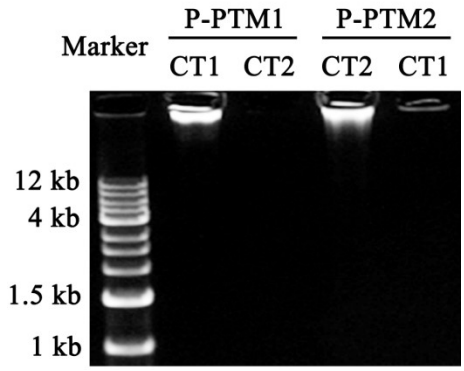


**Figure S5.** Gel electrophoresis characterization of the RCA products. Efficient RCA happen when DNA primer probes and corresponding circularized DNA templates react together. CT1 and CT2 are circularized template probes for PTM1 and PTM2.

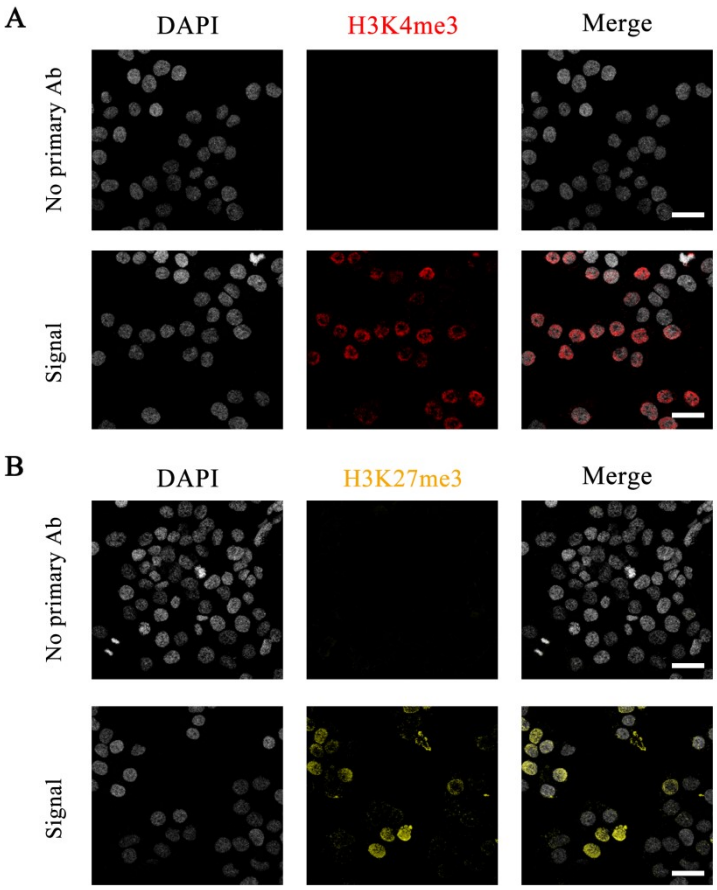


**Figure S6.** Cell images for RCA of individual PTM (A) H3K4me3 and (B) H3K27me3 in MCF-10A cells. Scale bar, 30 μm.

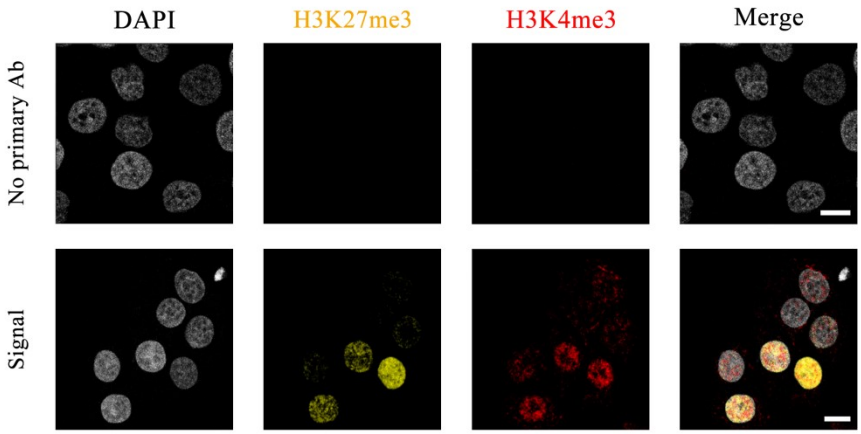


**Figure S7.** Simultaneous RCA for H3K4me3 and H3K27me3. The sample with no primary antibody was applied as negative control. Scale bar, 10 μm. MCF-10A cell line was used here.

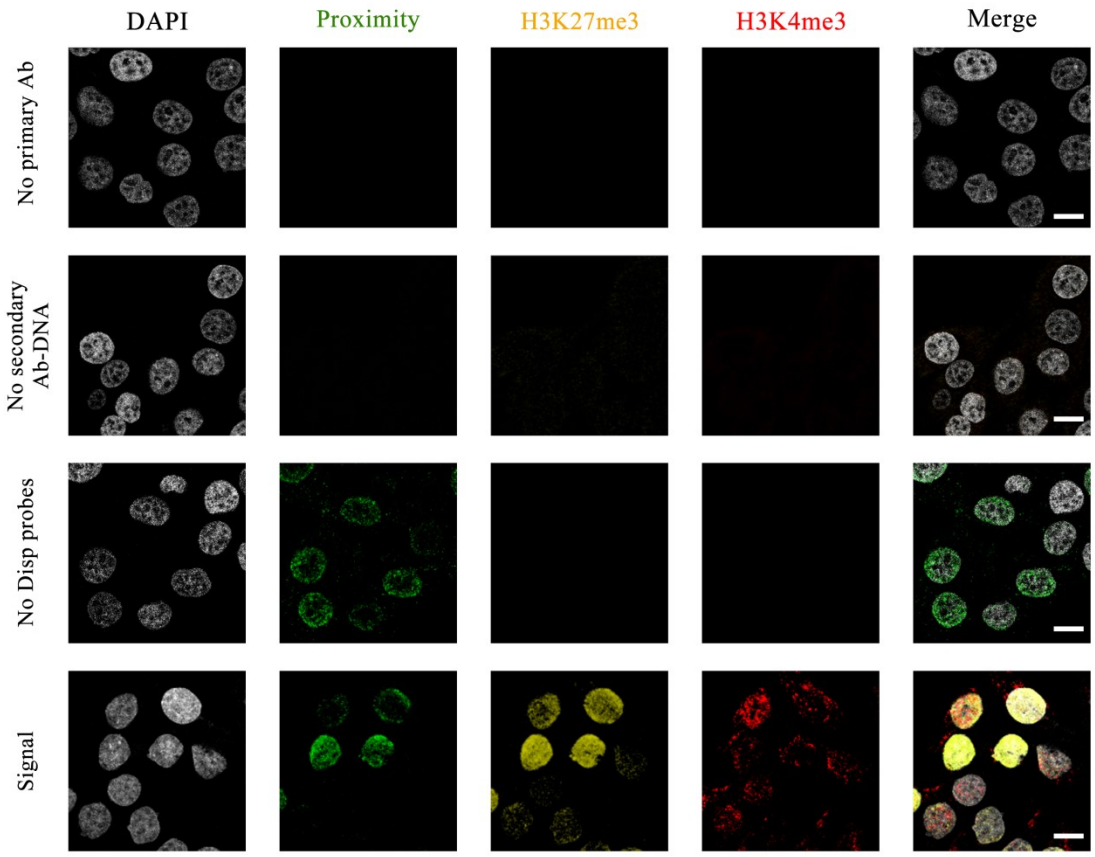


**Figure S8.** Exploration of the proximity of PTMs by PBA-based imaging. A number of negative controls were set to demonstrate the feasibility of the proposed strategy. "No Disp probes" refers to the experimental control that without the displacement probes. Scale bar, 10 μm. MCF-10A cell line was used.

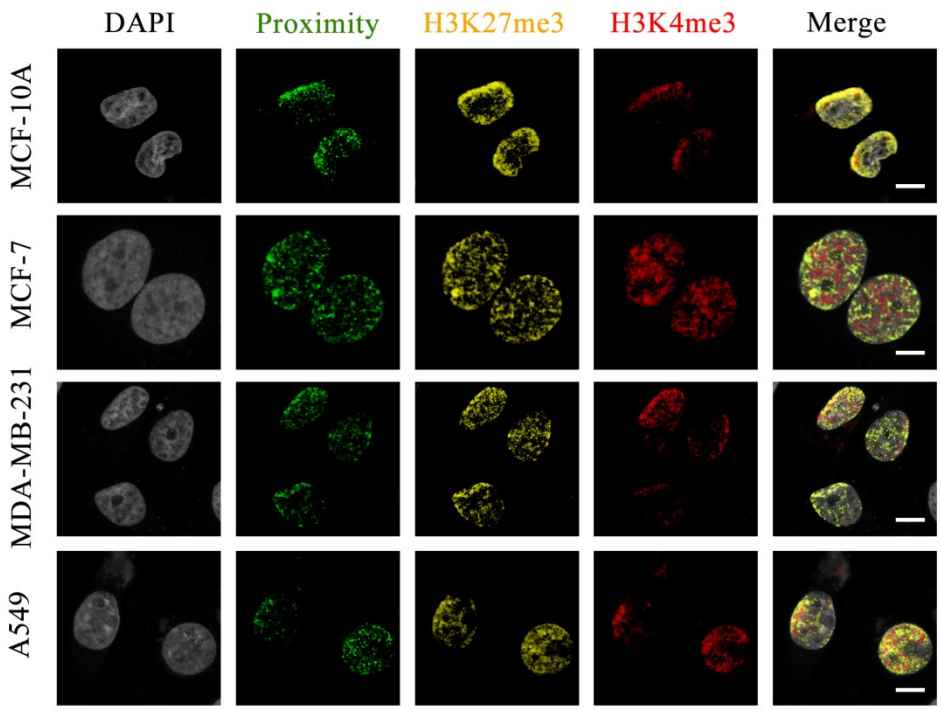


**Figure S9.** The investigation of individual and proximity sites of H3K4me3/H3K27me3 by PBA-based imaging in different cell lines. Scale bar, 5 μm.

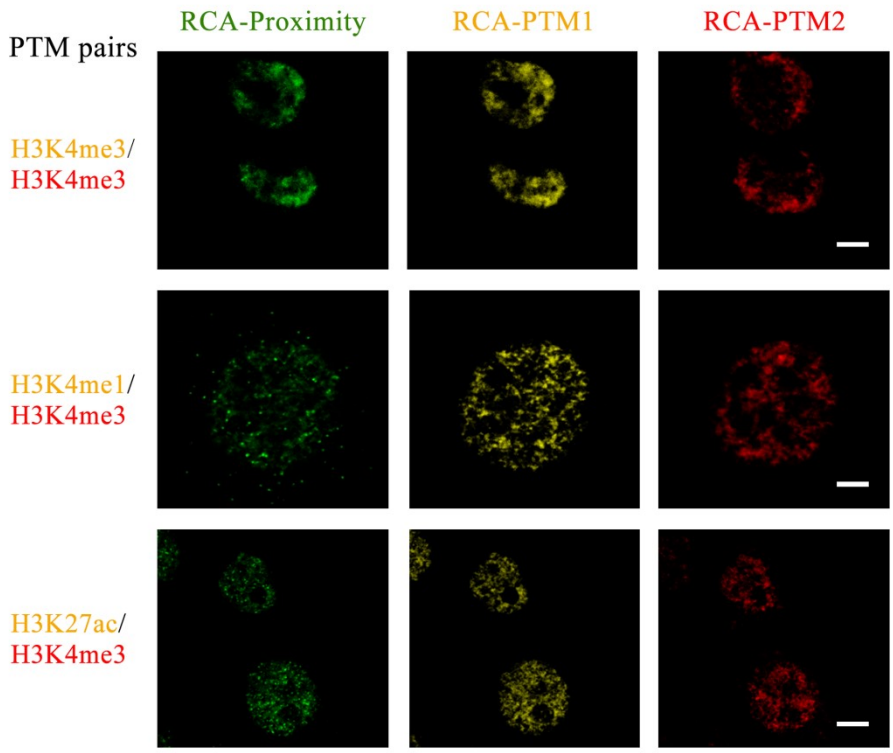


**Figure S10.** The simultaneous imaging of two individual PTMs and their spatial proximities. Different PTM pairs were imaged using PBA. Scale bar, 5 μm. MCF-10A cells were used in this experiment.

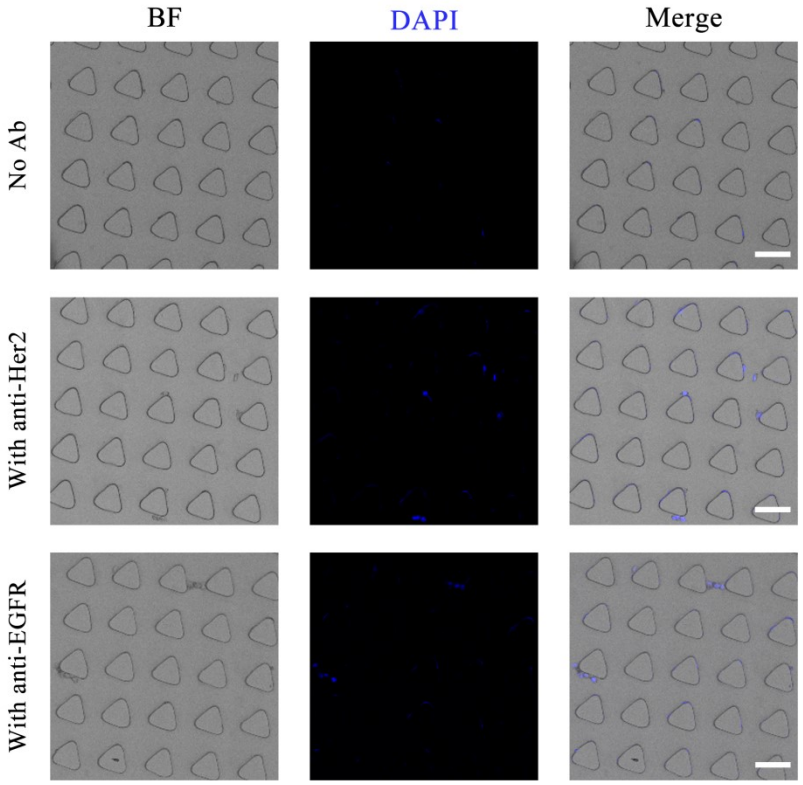


**Figure S11.** The capture of MDA-MB-231 cells in different immunocapture regions on the chip. The region with no antibody modification was not capable to capture any cells. Cells were specifically captured in Her2- and EGFR-antibody modified regions. Scale bar, 70 μm.

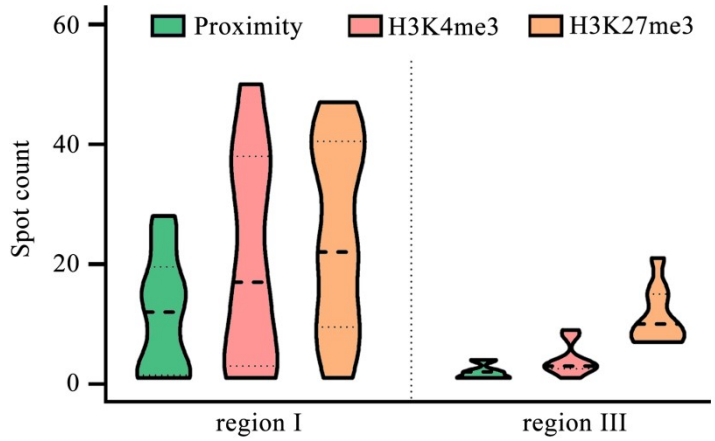


**Figure S12.** The statistical analysis of MDA-MB-231 cells in different immunocapture regions. Region I and III corresponds to anti-Her2- and anti-EGFR-modified regions in the microfluidic chip. No cells were captured in Region II with anti-PR modification. The numbers of analyzed cells are 9 (N=9).

REFERENCES

1 J. Xue, F. Chen, L. Su, X. Cao, M. Bai, Y. Zhao, C. Fan, Y. Zhao, Angew. Chem. Int. Ed. 2021, 60, 3428-3432.