Supporting Information

Plasmonic Nanoparticle-Assisted Single-Molecule Dynamic Binding for Protein Kinase Activity Digital Counting

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1. Experimental Methods

1.1 Chemical and materials

Chloroauric acid ($HAuCl_4 \cdot 3H_2O$) was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). 3-Aminopropyltriethoxysilane (APTES) was purchased from J&K Scientific, Ltd. (Beijing, China). Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), Adenosine triphosphate (ATP), ascorbic acid (AA), Sodium citrate and Tris- HCl were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). mPEG-succinimidyl valerate (mPEG-SVA, MW, 5000) were obtained from SeeBio Co. (Shanghai, China). Bovine serum albumin (BSA), and human serum albumin (HSA) were purchased from Aladdin (Shanghai, China). Protein kinase A (PKA), carboxypeptidase Y (CPY), glutathione (GSH), N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and glucose oxidase (GOX) were purchased from Sigma-Aldrich (U.S.A.). Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were purchased from Invitrogen Corp (Carlsbad, CA, U.S.A.). In the experiment, ultrapure water was used throughout the experiment. The peptide sequences used are as follows:
Capture Peptide: $CGGG(KIAALKE)_3S$
Signal Peptide: $(EIAALEK)_2K$-TAMRA

1.2 Synthesis of AuNPs by seed-mediated growth

The spherical gold nanoparticles (AuNPs) used in this experiment were synthesized based on a seed-mediated growth method.[1, 2] Herein, 18 nm AuNPs were used as the seed for the synthesis of larger size AuNPs. Briefly, 1.03 mL of 24.28 mM $HAuCl_4$ was mixed with 98.97 mL of DI water, and then the mixture was heated to 110 °C. As the temperature reached 110 °C, 10 mL of 1.455×10^{-2} M trisodium citrate was rapidly injected to the boiling solution. The mixture was vigorously stirred and refluxed for 20 min. The color of the mixture would gradually change from colorless to pale red, pale purple, and finally to the color of wine red. After the color stays the same, the colloidal solution was kept stirring at room temperature until the solution was cooled down. The colloidal solution was stored at 4 °C before use. Typically, to synthesize larger size AuNPs (60 nm), the as-prepared 18 nm AuNPs were used as the seed. 10 mL of the seed AuNPs solution was injected to a mixture including 40 mL DI water and 30 mL of 0.13 M trisodium citrate. The obtained mixture was then heated to 120 °C and followed by gradually adding 20 mL of 2.35×10^{-3} M $HAuCl_4$ to the hot solution for 40 min under gently stirring. As the reaction completed, the solution was kept stirring to cool down to room temperature and stored at 4 °C before use.

1.3 Slide preparation and total internal reflection fluorescence microscopic imaging

The glass slides cleaned sequentially with chromic acid lotion (boil in water bath for 40 min)，piranha solution (boil in 80 °C water bath for 90 min) and then were rinsed with ultrapure water. To fix AuNPs on the cover glass surface, the cleaned slides were functionalized with amination solution (1 μL APTES in 10 mL EtOH). Before microscopic imaging, vacuum grease was used to make flow

channels on the surface of the amino-modified glass slide. Sodium citrate-modified AuNPs with appropriate concentration were injected into the flow channel. The capture probes (CP, 1 μM) were injected into the AuNP-adsorbed slides and incubated overnight at 37 °C for 12 hours. Then the slides were washed by water and passivated by PEG-SVA for an hour. PKA was injected to the channel. The peptides were phosphorylated at the specific sites for 1 h. Finally, CPY was added to cleave the unphosphorylated capture CP for 0.5 h. The channel was further washed with Phosphate buffered saline (PBS).

Single-molecule fluorescence measurements were performed on a home-built imaging system based on an Eclipse Ti-U inverted epi-fluorescence microscope (Nikon). A tightly focused Gaussian laser beam (532 nm) was utilized to directly excite the fluorescence. The fluorescent signals were collected by a 100× TIRF objective (NA 1.49) and then captured by an EMCCD (iXon ultra 897, Andor, Belfast, United Kingdom). 2000 frames were captured to track the intensity trajectory and the frame rate was operated at 5 fps and gain was 4. All movies were processed and analyzed with MATLAB (MathWorks, Inc., Natick, MA) and Image J (National Institutes of Health, Bethesda, MD).

1.4 Detection of endogenous PKA in HeLa extract

At first, Hela cells were cultured in DMEM with 10% FBS and 1% PS at 37 °C with 5% CO_2. Then, the culture medium was replaced by 3.0 mL of serum-free medium, and the cells were further incubated for 4.0 h. To stimulate intracellular PKA, the cultured cells were treated with 10 μL of Forskolin/IBMX solution (freshly prepared with DMSO) with different concentrations for 30 min. As a control, DMSO alone was replaced with the drug solution for the unstimulated sample. The cultured cells were then collected by scraping and lysed in a nucleoprotein extraction kit. After that, the lysates were centrifuged at 12 000 rpm for 20 min at 4 °C, and the resulting supernatants were stored at -20 °C for further use.

1.5 Analysis of SiMREPS data

All raw images were first processed by imageJ. Then the fluorescence intensity-versus-time trajectories were extracted from acquired movies by custom MATLAB code. Single-molecule fluorescence traces displaying single-step photobleaching and a signal-to-noise ratio of > 3 were selected for kinetic analysis. The trajectories were fitted with Hidden Markov model (HMM) to identify the number of transitions and dwell times of the bound (τ_{on}) and unbound (τ_{off}) states from individual candidate molecules.

2. Supporting Figures

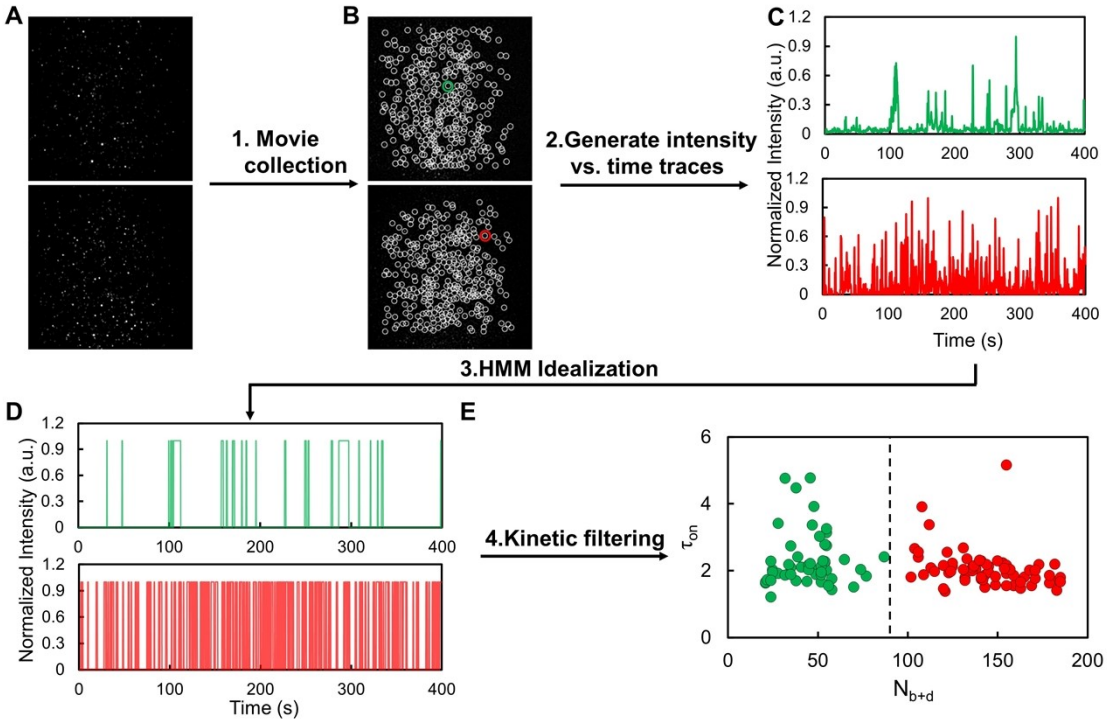

Figure S1. (A) Single-frame images of representative fields-of-view from TIRF microscopy. (B) Intensity fluctuation maps of the fields-of-view as shown in (A). White circles indicate the positions of local maxima in the fluctuation map, from which candidate region-of-intersts (ROIs) are identified for further analysis by generating the intensity *vs.* time traces. (C) Representative intensity *vs* time traces generated from the ROIs identified in (B), red represented positive group and green represented negative controls. (D) HMM idealization for each intensity *vs.* time trace. (E) Candidates in the positive (red circles) and negative (green squares) are well separated by the threshold, permitting discrimination of specific and nonspecific binding at the single-molecule level.

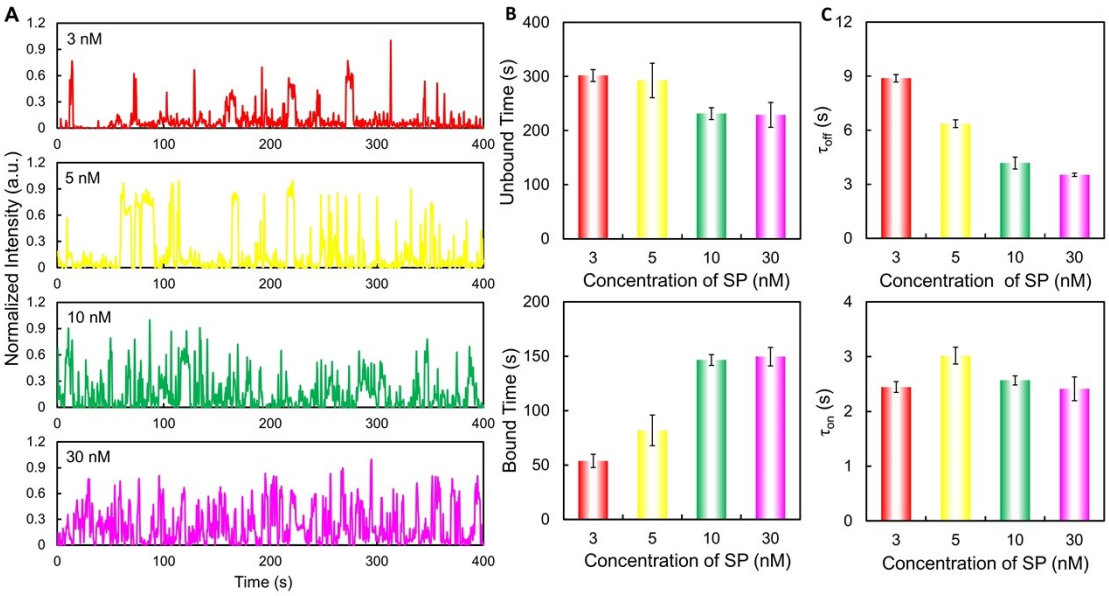

Figure S2. Influence of different concentrations of SP on the binding and dissociation kinetics. (A) Representative single-molecule fluorescence intensity-time trajectories at varying imager strand concentrations: 3, 5, 10 and 30 nM. (B) The total dwell time of unbound (top) and bound (bottom) states against different SP concentrations. (C) The average dwell time of the unbound (top) and bound (bottom) state at different SP concentrations.

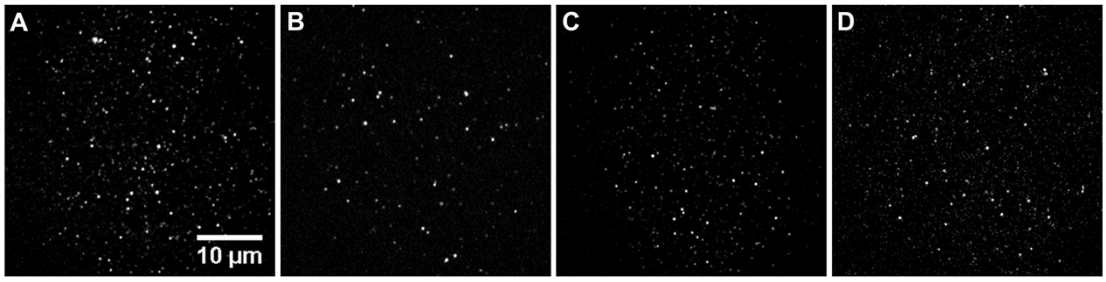

Figure S3. Effect of slide surface treatment on nonspecific binding of imager probes. Images show the extent of nonspecific binding of 3 nM TAMRA-labeled detection probe to coverslips passivated with 0.5 mg/mL BSA, (B) 0.5 mg/mL PEG-SVA, (C) 0.5 mg/mL PEG-SVA + 1 mM DST, (D) 0.5 mg/mL PEG-SVA + 1 mM DST + 0.5 mg/mL BSA.

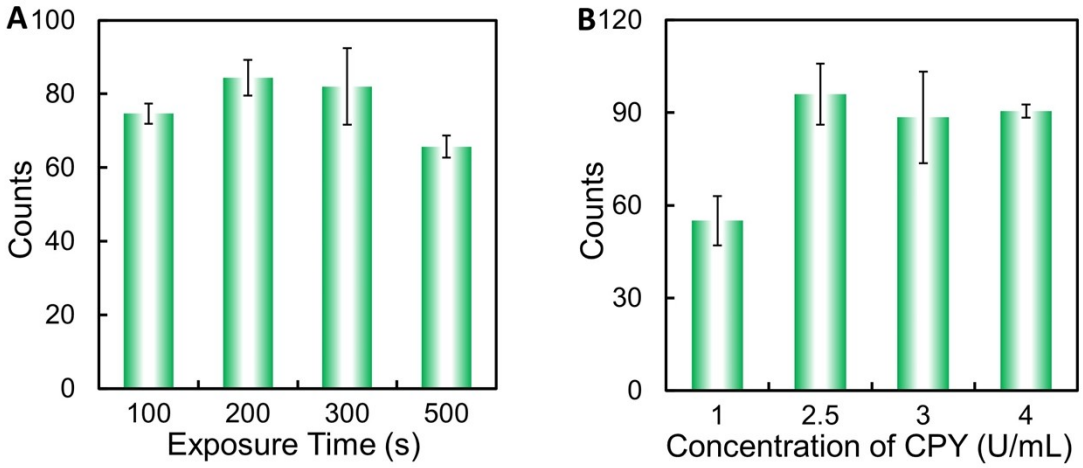

Figure S4. Optimization of assay condition for the PKA detection. (A) Effect of different exposure times on the single-molecule counting results obtained. (B) Different concentration of CPY against single-molecule counts. The concentrations of signal probe used in this assay is 5 nM.

3. References

1. Z. Ye, L. Wei, L. Xiao and J. Wang, *Chemical. Science.*, 2019, **10**, 5793-5800.
2. H. Liu, L. Wei, J. Hua, D. Chen, H. Meng, Z. Li and L. Xiao, *Nanoscale*, 2020, **12**, 10390-10398.