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Application of inhibitor-based probe to reveal the distribution of membrane PSMA in dSTORM imaging

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Experimental Section

Cell culture

LNCaP cells and Juekat cells were purchased from Shanghai Institute of Biological Sciences. Cells were grown in RPMI-1640 medium (Gibco) and supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C under a humidified atmosphere with 5% CO_2.

Ligand labeling

Conjugation of Alexa 647 and TAMRA dyes to anti-PSMA antibody, Alexa 647 to anti-FR and Alexa 647 to CD45 was performed by NHS-ester chemistry following the recommended procedures from the manufacturers (ATTO-TEC and Life Technologies). 0.5 μL Alexa 647 or TAMRA (Invitrogen, dissolved in DMSO, and the final concentration is 1 mg mL^{-1}) was mixed with 100 μL PSMA antibody (dissolved in PBS, and the final concentration is 200 μg mL^{-1}). The complex was shaken gently in the dark at 25 °C for 4 h. The redundant free dyes were removed using illustra NAP-5 columns (GE Healthcare). The UV/VIS spectrophotometer was used to test the labeling ratio of antibodies to dyes. We selected tubes with the binding ratio ranging from 0.8 to 1.

Staining and sample preparation

Cells were grown on a pre-cleaned coverslip (22 mm × 22 mm, Fisher) until they reached a coverage of 60–70%. Then cells were fixed with 4% Paraformaldehyde (PFA) with 0.2% glutaraldehyde (GA) for 10 min at room temperature, and washed out by PBS for three times. The

samples were blocked by incubating in 3% BSA for 10 min. Subsequently, cells were stained with 50 μl inhibitor probes for 10 min or TAMRA-conjugated PSMA antibodies for 30min in dark at room temperature, and washed out the staining solutions for three times with PBS again. For preparation of the smaples in dual-color imaging, the cells were treated as described above, except that they were labeled with two staining solutions simultaneously (Alexa 647-conjugated anti-PSMA and TAMRA-conjugated inhibitor probes or Alexa 647-conjugated anti-FR and TAMRA-conjugated inhibitor probes).

dSTORM imaging

Before performing dSTORM, the imaging buffer containing 10% (w/v) glucose, 0.5 mg/mL glucose oxidase (Sigma-Aldrich), 40 μg/mL catalase (Sigma-Aldrich) and 1% (v/v) beta-mercaptoethanol was fresh-prepared. Then 100 μL imaging buffer was added on a large coverslip (24 mm × 50 mm, Fisher), and the small coverslip (22 mm × 22 mm) containing cells was inversely sealed onto the large one using nail polish.

Samples were observed on a Nikon Ti-E microscope equipped with an oil immersion objective (100 × 1.49 NA lens, Nikon, Japan) and imaged on an electron-multiplying charge-coupled device (EMCCD) camera (Andor, Belfast, Northern Ireland). The total internal reflection fluorescence (TIRF) system was applied to realize the imaging of cell membrane. The sample was illuminated using a 532 nm laser source (~2 kW/cm^2) with moderately high powers. 5000 frames were usually captured for each cell, with a time interval of 30 ms. Obtaining one single dSTORM image usually took less than 4 minutes. During the acquisition time, the z-drift was eliminated by the means a perfect focus lock of the microscope.

For the dual-color imaging, the sample was first excited by a 639 nm laser (~2 kW/cm^2) to acquire a red channel, and then, it was imaged under the excitation of a 532 nm laser with adding a band-pass emission filter (FF01-595/ 34-25, semrock) to avoid the crosstalk of a red channel. Meanwhile, sample drift was corrected between these two channels using 100 nm microspheres (Invitrogen) as fiducials markers.

Image reconstruction

For super-resolution image reconstruction, a freely available plug-in for Image J named ThunderSTORM was used to analyze raw images. At first, the PSF candidates were selected using ‘wavelet B-Spline filter’ method (the default settings were used for the prefilter). Then, signals

were detected searching the local intensity maxima with 8-connected neighborhoods in each frame, which were fit using an integrated Gaussian point spread function. Finally, cross-correlation method was used to determine the shift between the first image and each of the subsequent images.

Resolution measurement

The resolution of our home-built instrument was measured by the Fourier Ring Correlation (FRC) map. The FRC map is calculated by ImageJ plugin NanoJ-SQUIRREL. It requires two super-resolution images of the same region to run. Firstly, the image sequences are separated into odd and even frames. The two frames combine to be an image stack. Next, we drag the stack into Image J and run '*Calculate FRC Map*' from the NanoJ-SQUIRREL menu. Then, it generates a FRC map. FRC analysis was run locally on each block and the FRC map provided an estimate of resolution across the whole image. FRC value changed from small to large with the color from blue to red. The resolution of the block which has the lowest FRC value represented the highest resolution in the image.

CBC analysis

CBC analysis was performed to show the co-localization of dual-color dSTORM data. The CBC values are also calculated by ImageJ plugin ThunderStorm. This method utilizes the coordinate information of each localization instead of the intensity-based approach which depends on the chosen post-processing parameters and suffers from a limited resolution. It takes into account the multiple switching cycles of each set of localizations by comparing the spatial distribution of the surrounding localizations from both species, and avoids false positive colocalization when one of the molecular species is randomly distributed. The value of −1 indicates anti-correlated distribution, 0 means non-correlated distribution (no colocalization), and 1 represents a high probability of colocalization.

Cluster analysis by SR-tesseler

Herein, the software named SR-Tesseler was employed to analyze clusters as previous work has described. The dSTORM data were firstly imported into SR-Tesseler in the format of "csv". Then the cell region was circled as a ROI (region of interest) and polygons were created inside the ROI (Fig. S7a and S7b). Once the polygons have been reconstructed and the parameters have been computed. Next, the regions whose density δ^1_i satisfied the criterion $\delta^1_i > \delta$ were defined as

objects (Fig. S7c). The object outlines were circled by connecting all localizations belonging to the borders of the objects. Finally, clusters were extracted within the objects in which the density of localizations was higher than the average density of the objects (Fig. S7d).

Tessellation-Based Colocalization Analysis.

The tessellation-based colocalization analysis is performed by Coloc-Tesseler software, allowing us to investigate the degree of colocalization between two channels. The dual-color dSTORM data were first imported into the Coloc-Tesseler software, and the merged image was generated by clicking on the 'Colocalization' icon. Then a scatterplot was exported and the colocalization value (Manders or Spearman rank coefficients) was automatically computed. The scatterplot is depicted as a 2D histogram, always keeping the density of the red channel in abscises and the density of the green channel in ordinates. Thirty regions with an area of 5×5 μm^2 were analyzed and the Manders or Spearman rank coefficients were presented as mean ± Stand

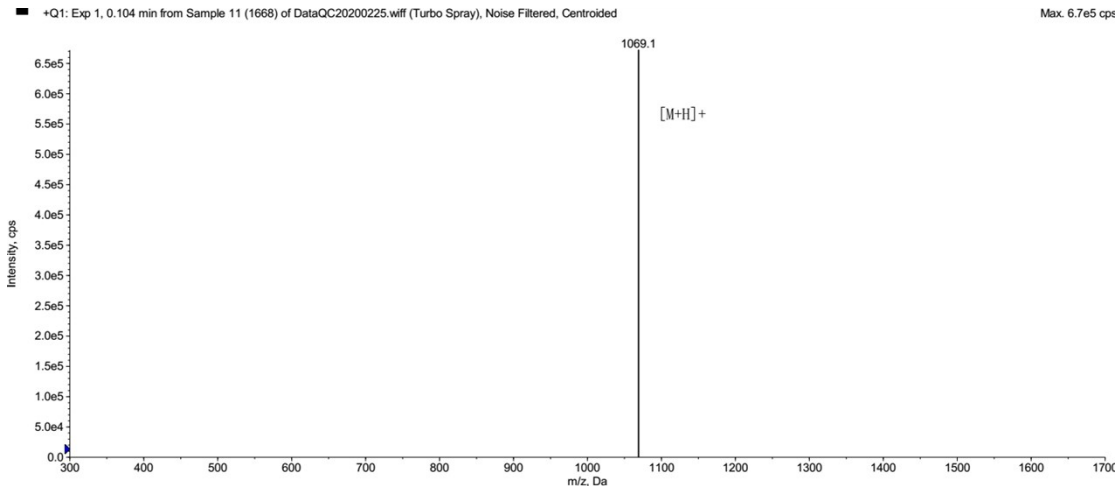

Fig. S1 Mass spectrum of TAMRA-conjugated PSMA617 linker.

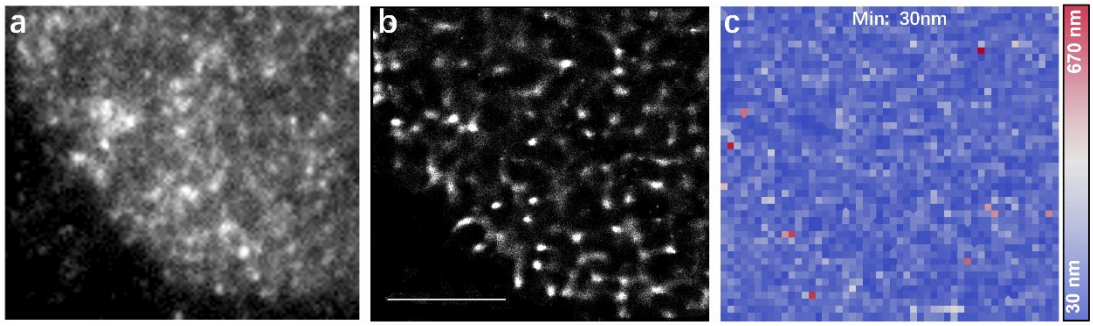

Fig. S2 Conventional fluorescent imaging (a) and dSTORM imaging (b) of PSMA on LNCaP cell membrane. The conventional fluorescence image (a) shows the diffraction-limited distribution of PSMA, while dSTORM imaging improves the resolution significantly (b). (c) The corresponding FRC map. The minimum value of the block represented the highest resolution in the image, which was 30 nm. Scale bars, 5 μm.

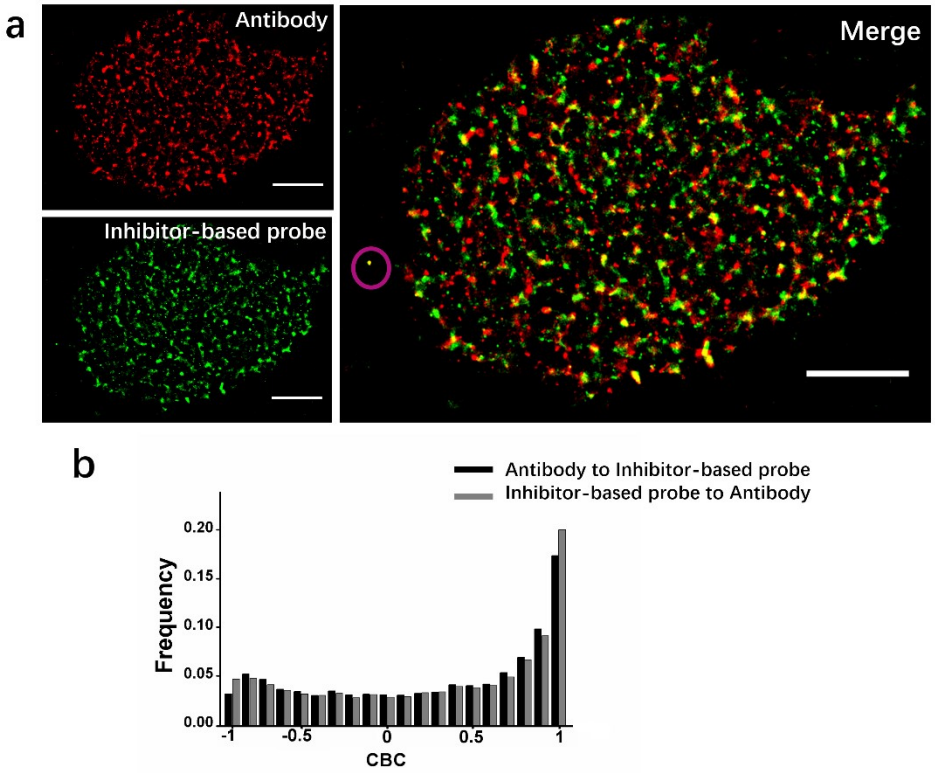

Fig. S3 Dual-color imaging of PSMA labeled with two different probes on the apical membrane. (a) The PSMA on LNCap cell membrane labeled by antibody-Alexa647 (red) and inhibitor-TAMRA (green), respectively. The superimposed image indicates significant colocalization between these two channels. Scale bars, 5 μm. Microspheres were used as fiducial markers to correct the x–y drift and the optical registration between 647 nm and 532 nm channels. (b) The histogram of the colocalization parameter for these two probes by CBC analysis. Data were from ten full-sized cells.

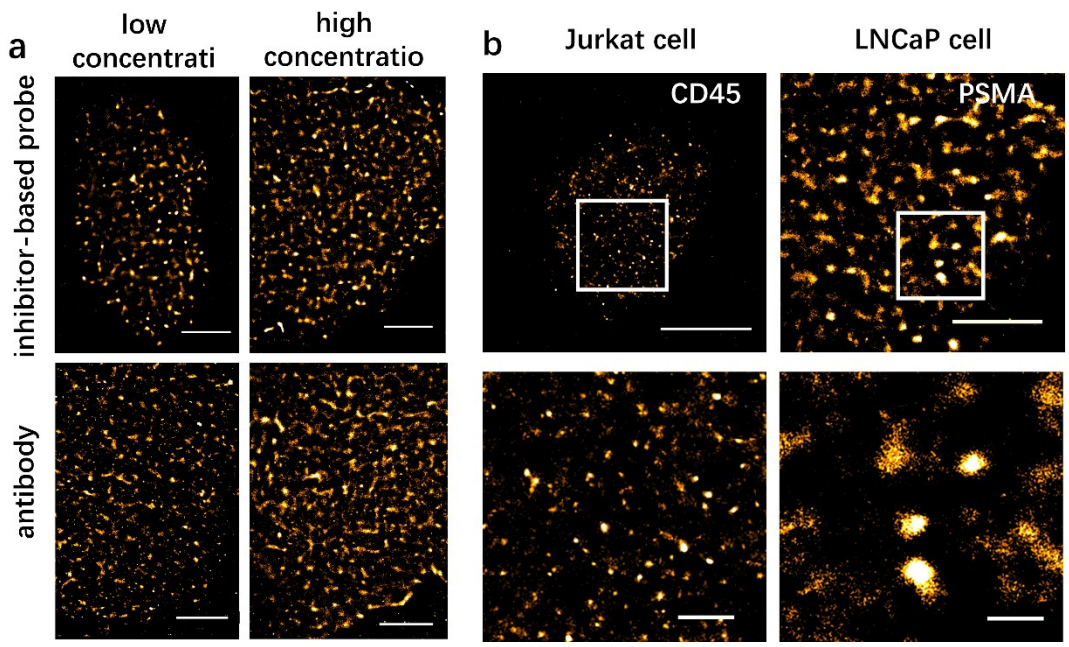

Fig. S4 The different spatial distribution of PSMA and CD45 on the cell membranes. (a) PSMA is labeled with a low or a high concentration of inhibitor probes (0.005 μg/mL and 0.2 μg/mL) and antibodies (0.05 μg/mL and 0.15 μg/mL) respectively, showing its clustered distribution. Scale bars indicate 5 μm. (b) The representative dSTORM images and corresponding magnified regions of CD45 and PSMA. The CD45 proteins show a homogeneous distribution on Jurkat cell membrane and PSMA proteins form clusters on LNCaP cell membrane. Scale bars: 5 μm in the original images and 1 μm in the magnified images.

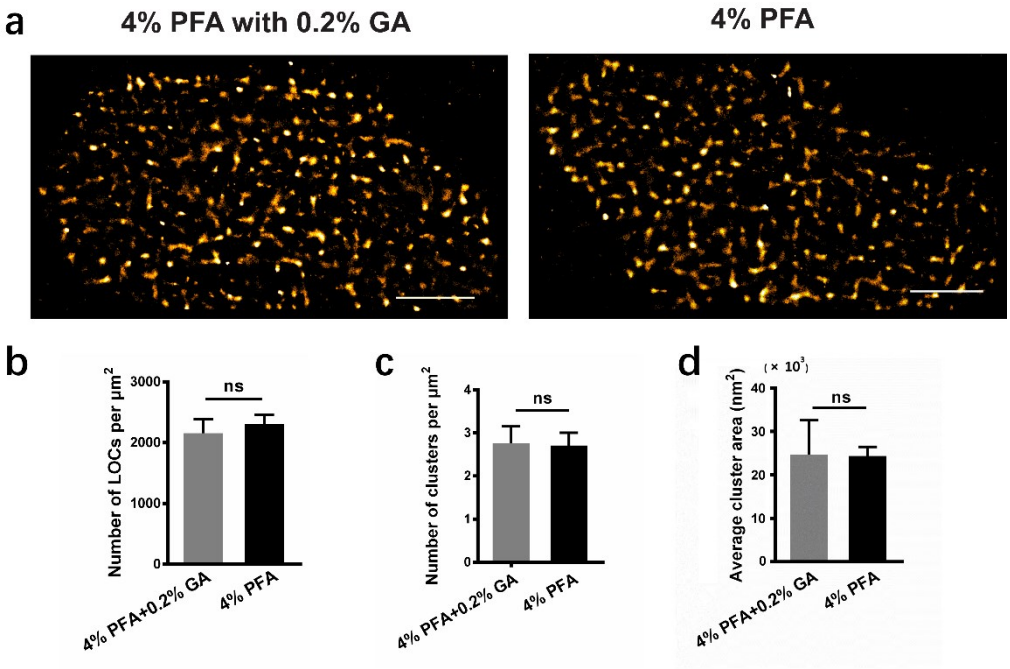

Fig. S5 The influence of different fixing solutions on dSTORM imaging. (a) The representative dSTORM images of PSMA fixed by 4% PFA with 0.2% GA or 4% PFA, respectively. Scale bars indicate 5 μm. (b-d) Quantitative comparisons of the parameters under the two fixation conditions: the number of localizations per μm^2 (b), the number of clusters per μm^2 (c) and the average cluster area (d). All data are shown as the means ± standard deviation (s.d.). "ns" means no significance (two-tailed unpaired t test).

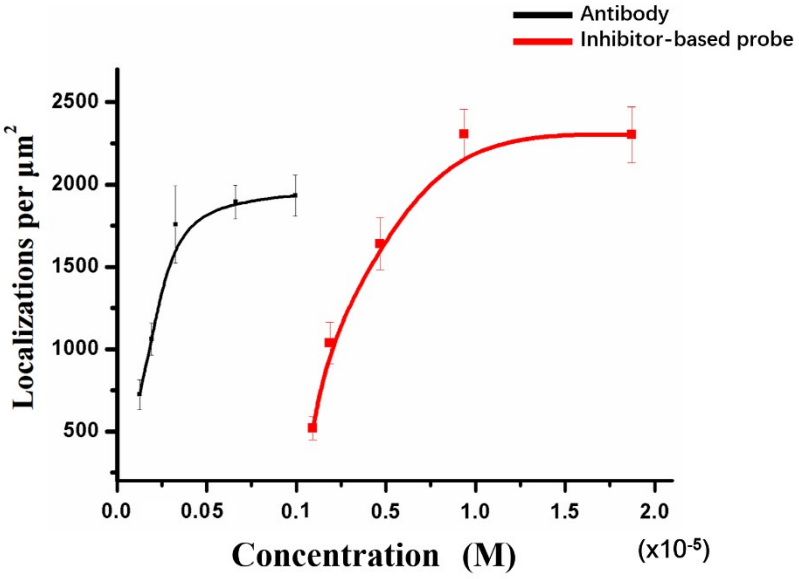

Fig. S6 The saturated concentration curve of antibody and inhibitor-based probes for labeling PSMA on LNCaP cell membrane by calculating the number of localizations per μm^2. Data shown are means ± standard deviation (s.d.). Ten cells in three independent experiments were collected for each concentration.

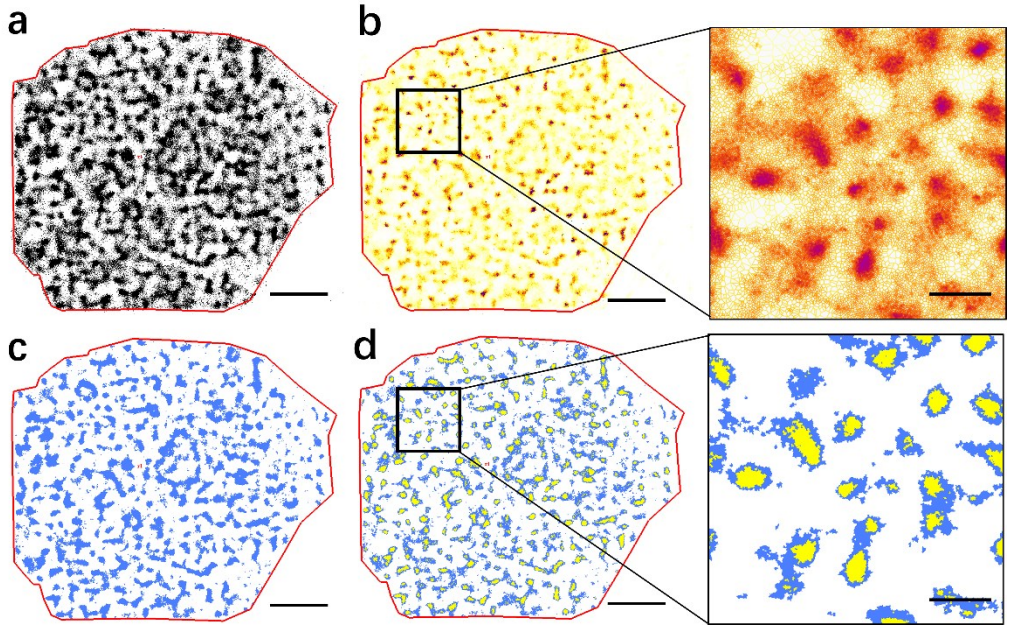

Fig. S7 The illustration of SR-Tesseler method. (a) Representative localization map of PSMA. The cell membrane is circled by red lines. (b) Segmentation of the localizations by bisectors between the nearest localizations. The enlarged one shows the detail information. (c) Identification of objects (blue) after thresholding the object density $\delta^1_i > \delta$. (d) The extracted clusters (yellow regions) with a higher localization density than the objects. Scale bars: 5 μm in original images and 1 μm in magnified images.

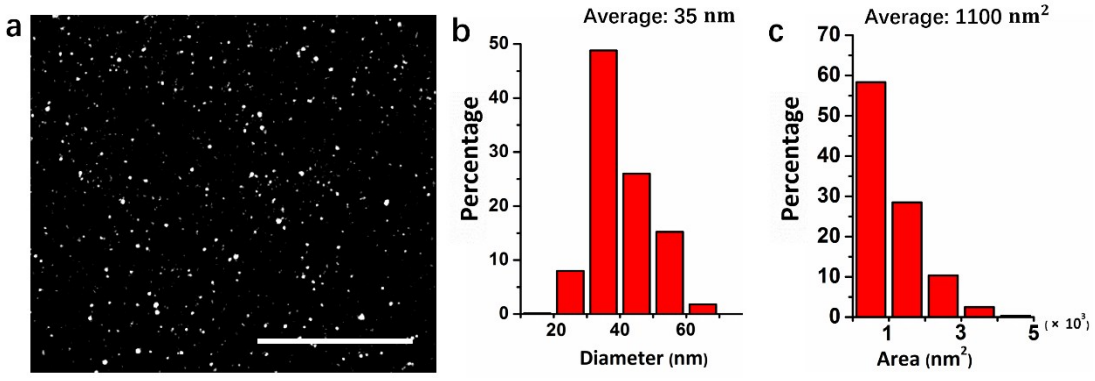

Fig. S8 Characterization of a single emitter by SR-Tesseler method. (a) A representative dSTORM image of inhibitor-based probes on an empty coverslip. Scale bar, 5 μm. (b-c) Quantitative analysis of the diameter (b) and area (c) of single blinking molecules. Statistics were from ten cells in three independent experiments.