

Supporting information

Developing substrate-based small molecule fluorescent probes for super-resolution fluorescent imaging of various membrane transporters

Qiang Wu, Mingjun Cai, Jing Gao, Tan Zhao, Haijiao Xu, Qiuyan Yan, Yingying Jing,
Yan Shi, Chuanqing Kang*, Yi Liu, Feng Liang, Junling Chen*, and Hongda Wang*

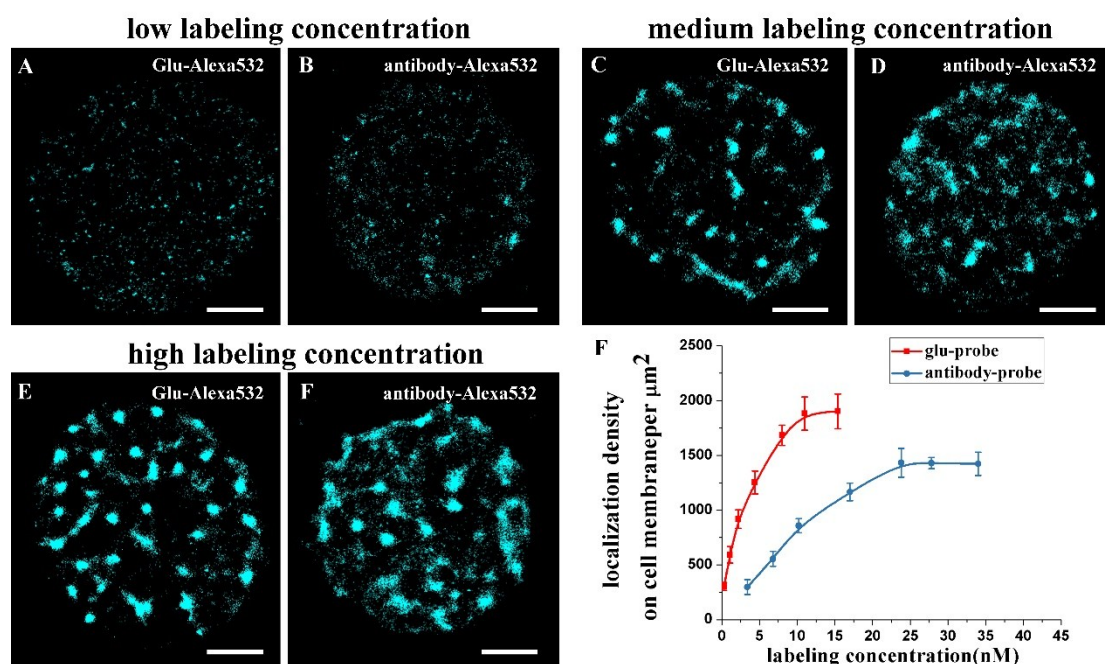


Figure S1. Saturated binding of Glu-Alexa532 and antibody-Alexa532 to GLUT1 on the RBC membranes. A-F) A series of dSTORM reconstructed images of GLUT1 on the RBC membranes under the increasing labeling concentration of Glu-Alexa532 (left) and antibody-Alexa532 (right). Scale bars are 2 μm in A-F. G) The concentration gradient labeling curves were plotted to determine the saturation labeling concentration of these two kinds of fluorescent probes.

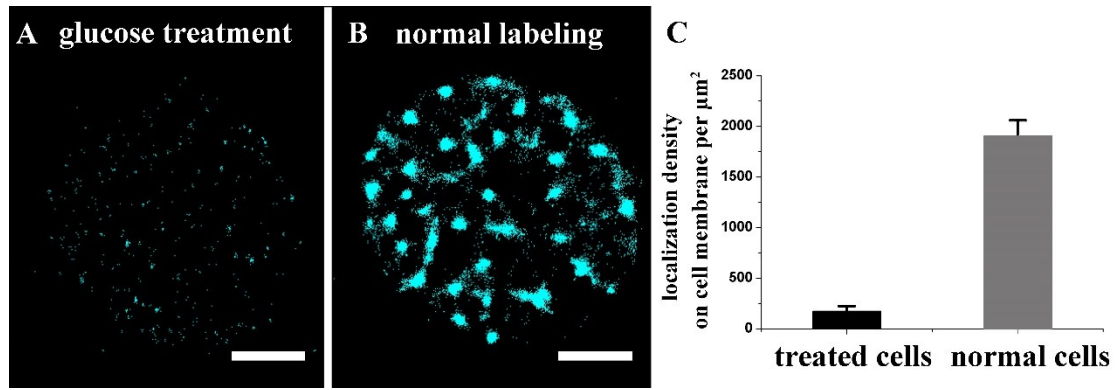


Figure S2. Free glucose can specifically replace the Glu-probe from the binding site of glucose transporter on the RBC membrane. A and B) The dSTORM imaging of GLUT1 on the RBC membranes treated with free glucose (A) or not (B) after being labeled by Glu-Alexa532. Scale bars are 2 μm . C) The statistical histogram of localization density on the RBC membranes with glu-replacement and normal labeling.

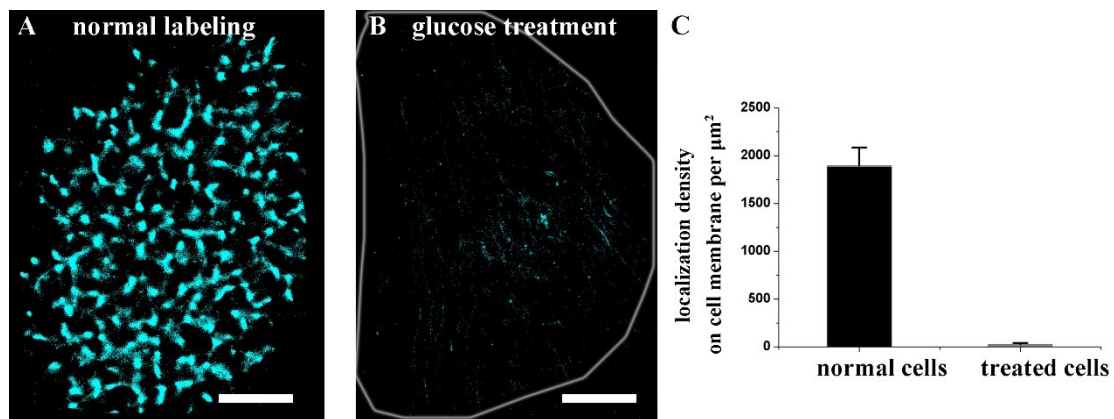


Figure S3. Free glucose can specifically replace the Glu-probe from the binding site of glucose transporter on the HeLa membrane. A and B) The dSTORM imaging of glucose transporters on the HeLa membranes treated with free glucose (B) or not (A) after being labeled by Glu-based probe. Scale bars are 5 μm . C) The statistical histogram of localization density on the HeLa membranes with normal labeling and glu-replacement.

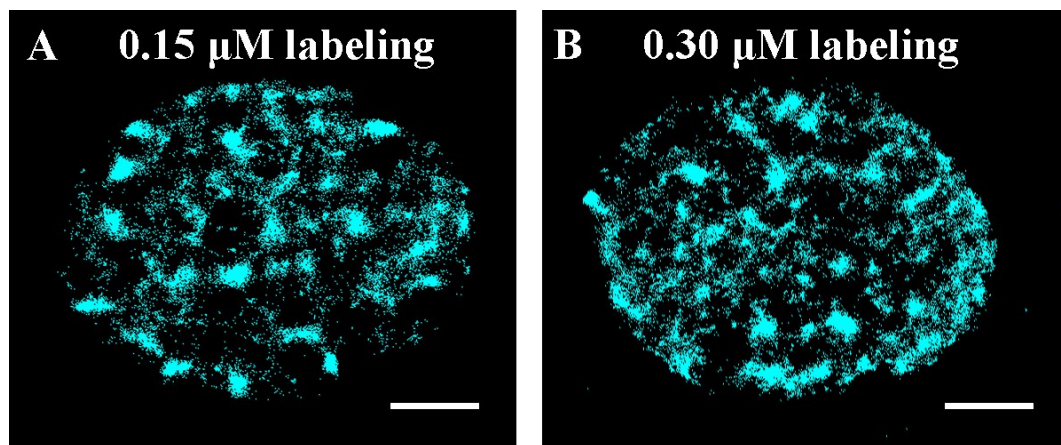


Figure S4. dSTORM imaging of Tyr transporters on the RBC membrane under (over-)saturated labeling concentration of Tyr-TAMRA. The similar dSTORM images of Tyr transporters on the RBC membrane under 0.15 μM (A) and 0.30 μM (B) labeling concentration. Scale bars are 2 μm .

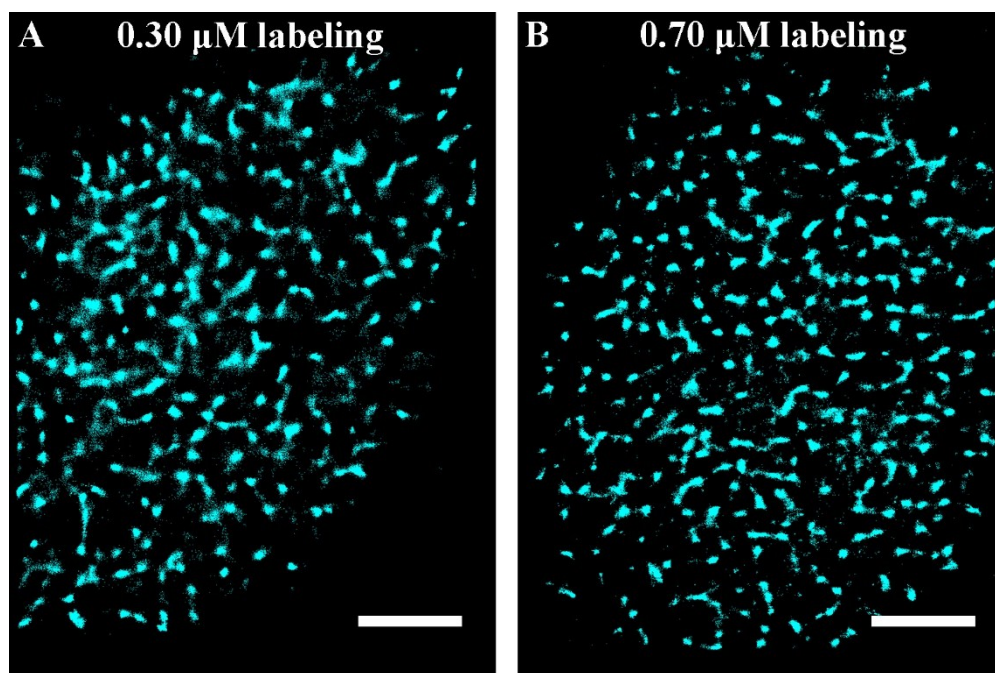


Figure S5. dSTORM imaging of Tyr transporters on the HeLa membrane under (over-)saturated labeling concentration of Tyr-TAMRA. The similar representative dSTORM images of Tyr transporters on the HeLa membrane under 0.30 μM (A) and 0.70 μM (B) labeling concentration of Tyr-TAMRA. Scale bars are 5 μm .

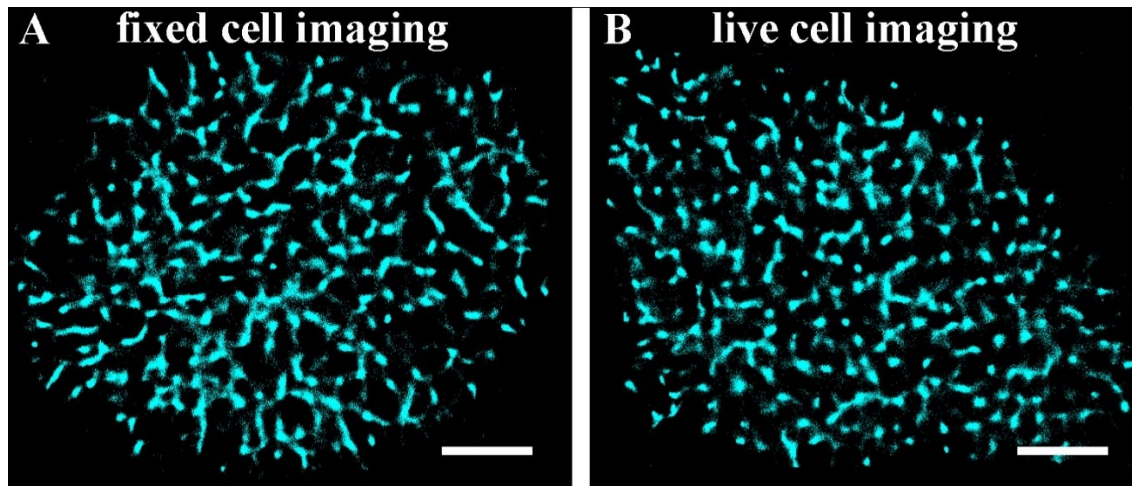


Figure S6. The similar dSTORM reconstruction images of Tyr transporters on the fixed Hela membrane (A) and live Hela membranes (B) under the same labeling concentration of Tyr-TAMRA. Scale bars are 5 μm .

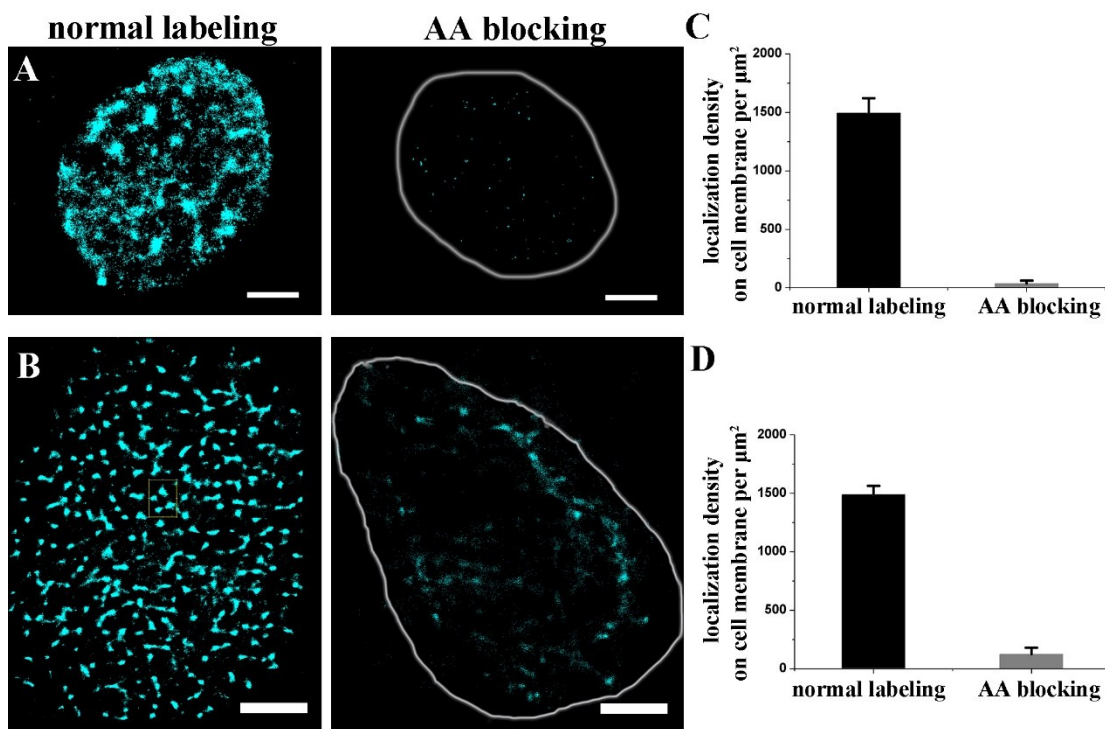
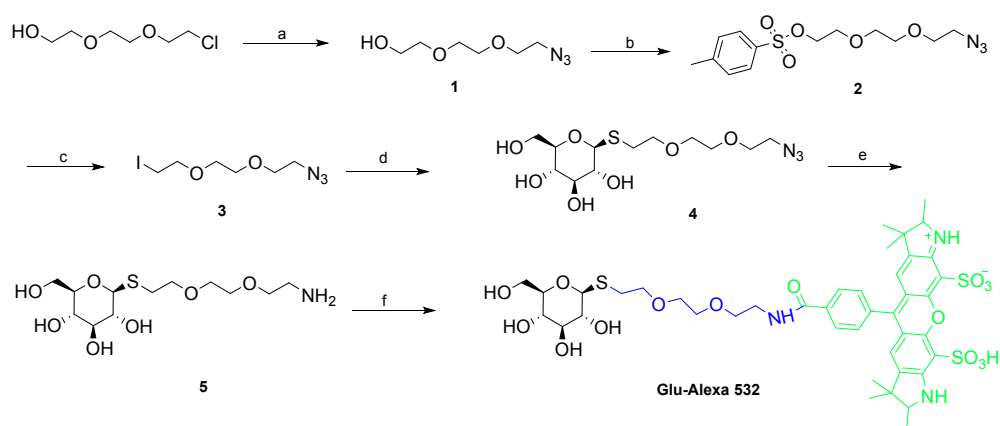


Figure S7. High specificity of Tyr probe is confirmed by the competition binding experiment of free Phenylalanine on the RBC and Hela membrane. A-D) The dSTORM imaging of Tyr transporters on the RBC (A) and Hela (B) membranes stained with Tyr-probes in the absence (left) or presence (right) of free Phenylalanine. Scale bars are 5 μm . C and D) The statistical histogram of localization density on the RBC (C) and Hela (D) membranes with normal labeling and AA blocking.

Experimental Methods

1. Synthesis of Glu-probe



Scheme 1: Synthesis route of Glu-Alexa532. Reagents and conditions: a) NaN_3 , DMF, 90°C , overnight; b) TsCl , TEA, DCM, RT, 12 h; c) NaI , DMF, 50°C , 5 h; d) 1-Thio-Beta-D-Glucose Sodium Salt, DMF, 50°C , 2 h; e) H_2 , Pd/C , MeOH, 2 h; f) Alexa Fluor 532, DMF, RT, dark, overnight.

General Methods: substrates and reagents are commercially available, and used as received. ^1H and ^{13}C spectra were recorded with Bruker AVX 400 MHz spectrometers in CDCl_3 and D_2O . Tetramethylsilane ($\delta = 0$ ppm) was used as internal standard for ^1H and ^{13}C spectra. The structures of known compounds were confirmed by ^1H NMR spectroscopy and comparison with literature data.

2-(2-(2-Azidoethoxy)ethoxy)ethanol (1)

2-(2-(2-Chloroethoxy)ethoxy)ethanol (5.0 g, 30 mmol) and sodium azide (3.9 g, 60 mmol) were dissolved in dry DMF (40 mL). The mixture was stirred at 90°C overnight. The solvent was evaporated under reduced pressure. Then the residue was washed with water and extracted with ethyl acetate (EtOAc) for three times. The organic phase was combined, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure to afford 2-(2-(2-Azidoethoxy)ethoxy)ethanol (5.0 g) as a colorless oil. The crude product was used for the next step without further purification. ^1H NMR (400 MHz, CDCl_3): δ 3.74 (t, 2H, $J = 4.4$ Hz), 3.70–3.68 (m, 6 H), 3.62 (t, 2H, $J = 4.4$ Hz), 3.40 (t, 2 H, $J = 5.0$ Hz), 2.30 (s, 1 H).

2-(2-(2-Azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (2)

To a stirred solution of compound 1 (4.0 g, 22.8 mmol) and TEA (4.62 g, 45.6 mmol) in DCM (40 mL) at 0°C was added tosyl chloride (6.52 g, 34.2 mmol) by portions. Then the reaction mixture was allowed to warm up to room temperature and stirred for 12 h. The mixture was washed with water and extracted by DCM for three

times. The organic phase was combined, washed with 1 M HCl, brine, dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by column chromatography with eluent (PE/EA = 4:1, v/v) to afford 2-(2-(2-Azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (6.0 g, 80%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃): δ 7.80 (d, 2H, *J* = 8.0 Hz), 7.35 (t, 2H, *J* = 8.0 Hz), 4.16 (t, 2H, *J* = 4.8 Hz), 3.70 (t, 2H, *J* = 4.8 Hz), 3.64 (t, 2H, *J* = 5.0 Hz), 3.60 (s, 4H), 3.36 (t, 2H, *J* = 5.0 Hz), 2.45 (s, 3H).

1-Azido-2-(2-(2-iodoethoxy)ethoxy)ethane (3)

To a stirred solution of compound 2 (2.0 g, 6.08 mmol) in dry DMF (10 mL) was added sodium iodide (2.73 g, 18.24 mmol), then the resulting solution was stirred at 50°C for 5 h. The solvent was evaporated under reduced pressure. The residue was washed with water and extracted with EA for three times. The organic layer was combined, washed with brine, dried over Na₂SO₄ and concentrated. The residue was purified by column chromatography with eluent (PE/EA = 6:1, v/v) to afford 1-Azido-2-(2-(2-iodoethoxy)ethoxy)ethane (1.30 g, 75%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃): δ 3.77 (t, 2H, *J* = 6.8 Hz), 3.71–3.68 (m, 6H), 3.40 (t, 2H, *J* = 5.0 Hz), 3.27 (t, 2H, *J* = 6.8 Hz).

Compound 4

Compound 3 (143.0 mg, 0.5 mmol) and Beta-D-Glucose Sodium Salt (131.0 mg, 0.6 mmol) were dissolved in dry DMF (2 mL), then the reaction mixture was stirred at 50°C for 2 h. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography with eluent (DCM/MeOH = 20:1, v/v) to afford compound 4 (120 mg, 68%) as a light yellow oil. ¹H NMR (400 MHz, D₂O): δ 4.51 (d, 1H, *J* = 9.8 Hz), 3.84 (d, 1H, *J* = 12.3 Hz), 3.77–3.61 (m, 10H), 3.46–3.35 (m, 5H), 2.99–2.80 (m, 2H). HRMS: calcd. for C₁₂H₂₃N₃O₇S [M+Na]⁺ 376.11489; found 376.11485.

Compound 5

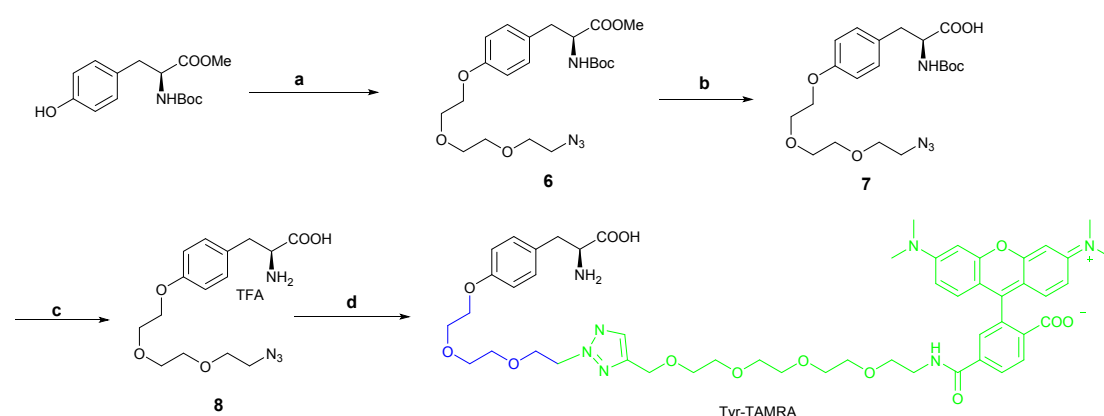
Compound 4 (60 mg, 0.17 mmol) was dissolved in dry MeOH (3 mL), Pd/C (20 mg) was added. The flask was degassed with nitrogen for three times, then filled with H₂, the resulting solution was stirred at room temperature for 2 h. The reaction mixture was filtered through celite and washed with MeOH for three times, concentrated under reduced pressure to give the compound 5 (40 mg) as a light yellow oil. The crude product was used for the next step without further purification.

Linkage the modified glucose to Alexa532

For the conjugation, 85 µg Alexa Fluor 532 was dissolved in 10 µL dry DMF and

add to 90 μ L compound **5** diluted solution (0.2 mg/mL, in dry DMF), then mixed thoroughly. The resulting solution was reacted in the dark at RT overnight with constant shaking. The DMF was evaporated under reduced pressure, the residue was purified by illustra NAP-5 columns (GE Healthcare) with phosphate-buffered isotonic saline (PBS) as eluent to afford the desired Glu-Alexa 532. The combination ratio of dye to glucose was determined by UV-visible absorption spectroscopy assay. Finally, the qualified solution with a suitable ratio (~ 1.0) were collected for labeling experiment.

2. Synthesis of Tyr-probe



Scheme 1: Synthesis route of Tyr-TAMRA. a) 1-Azido-2-(2-(2-iodoethoxy)ethoxy)ethane (**3**), K₂CO₃, DMF, 50 °C, 10 h; b) NaOH, MeOH/H₂O (v/v = 3:1), rt, 16 h; c) DCM/TFA (v/v = 5:1), rt, 4 h; d) CuSO₄, TCEP, TBTA, PBS, rt, 12 h, dark.

Compound 6

To a stirred solution of Methyl (tert-butoxycarbonyl)-L-tyrosinate (300 mg, 1.02 mmol) in DMF (5 mL) were added 1-Azido-2-(2-(2-iodoethoxy)ethoxy)ethane (214 mg, 1.22 mmol) and K₂CO₃ (423 mg, 3.06 mmol), then the resulting solution was stirred at 50 °C for 10 h. The solvent was evaporated under reduced pressure, then washed with water and extracted with ethyl acetate (EtOAc) for three times. The organic phase was combined, washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by column chromatography with eluent (PE/EA = 4:1, v/v) to afford compound **6** (400 mg, 88%) as a light yellow oil. ¹H NMR (400 MHz, CDCl₃): δ 7.03 (d, 2H, J = 8.0 Hz), 6.84 (m, 2 H), 4.96 (d, 2H, J = 8.0 Hz), 4.54 (d, 2 H, J = 8.0 Hz), 4.11 (m, 2 H), 3.86 (m, 2 H), 3.71 (m, 9 H), 3.39 (t, 2H, J = 4.0 Hz), 3.02 (m, 2 H), 1.42 (s, 9 H). ¹³C NMR (100 MHz, CDCl₃) δ 171.99, 157.47, 129.84, 127.76, 114.29, 79.46, 70.47, 70.35, 69.69, 69.44, 67.01, 54.12, 51.75, 50.29, 37.07, 27.89. HRMS: calcd. for C₂₁H₃₂N₄O₇ [M+H]⁺ 453.2344; found 453.2341.

Compound 7

To a stirred solution of compound 6 (400 mg, 0.88 mmol) in MeOH (3 mL) and H₂O (1 mL) was added NaOH (283 mg, 7.04 mmol), then the reaction mixture was stirred at room temperature for 16 h. The solvent was evaporated under reduced pressure, then the residue was redissolved in water (10 mL), the mixture was acidified with HCl (1.0 M), then extracted with ethyl acetate (EtOAc) for three times. The organic phase was combined, washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure to afford compound 7 (330 mg) as a light brown oil. The crude product was used for next step without further purification. ¹H NMR (400 MHz, CDCl₃): δ 7.08 (d, 2H, *J* = 8.0 Hz), 6.85 (m, 2 H), 4.92 (d, 2H, *J* = 8.0 Hz), 4.54 (d, 2 H, *J* = 4.0 Hz), 4.11 (t, 2 H, *J* = 4.0 Hz), 3.86 (m, 2 H), 3.70 (m, 6 H), 3.38 (t, 2H, *J* = 4.0 Hz), 3.10 (m, 2 H), 1.42 (s, 9 H).

Compound 8

Compound 7 (330 mg) was dissolved in DCM (5 mL) and TFA (1 mL), then the reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure to afford compound 8 (250 mg) as a light brown oil. The crude product was used for next step without further purification. ¹H NMR (400 MHz, *d*₆-DMSO): δ 8.24 (m, 3H), 7.16 (m, 2 H), 6.91 (m, 2 H), 4.13 (m, 1H), 4.07 (m, 2 H), 3.75 (m, 2 H), 3.60 (m, 6 H), 3.39 (m, 2 H), 3.03 (m, 2 H). HRMS: calcd. for C₁₅H₂₂N₄O₅ [M+H]⁺ 339.1663; found 339.1682.

Tyr-TAMRA

In 100 μ L of PBS, TAMRA-alkyne (4 mM) and compound 8 (4 mM) were allowed to react in the presence of CuSO₄ (1 mM), tris(carboxyethyl)phosphine (1 mM), and ligand (2 mM). After reaction at dark for 12 hours, the reaction mixture was purified by illustra NAP-5 columns (GE Healthcare) with PBS as eluent to obtain the desired Glu-Alexa 532. The combination ratio of dye to Tyr was measured by UV-visible absorption spectroscopy assay. Finally, the qualified solution with a suitable ratio (~1.0) were collected for labeling experiment.

3. Linkage of antibody to organic dye

Stock solution of GLUT1 antibody was diluted to the appropriate concentration with PBS (commonly, 100 μ g/ mL, 100 μ L), then added 0.2 μ L Alexa532 (or Alexa647) (1mg/mL, Invitrogen) to react with antibody in the dark for 2h at RT under continuous vortex. After reaction, the excessive free dyes were removed by filtration with illustra NAP-5 columns (GE Healthcare). The combination ratio of dye to antibody was determined by UV-visible absorption spectroscopy assay. Finally, the qualified solution with a suitable ratio (0.7-1.5) were collected for labeling experiment.

4. Cell culture

The Hela cells were purchased from Stem Cell Bank, Chinese Academy of Sciences. They were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone) with 10% fetal bovine serum (FBS, HyClone), 100 U/mL penicillin and 100 µg/mL streptomycin, in a 5% CO₂ humidified incubator at 37°C. For dSTORM imaging, the cells were cultured on a clean cover slip (22 mm × 22 mm, Fisher) in a dish for at least 24 hours to achieve ~60-70% confluence.

5. Sample preparation of dSTORM imaging

5.1 For dSTORM imaging of RBCs

Fresh red blood cells (RBCs) were obtained from centrifuging the whole human fingertip blood of healthy donors. In details, RBCs were washed five times with PBS by a low speed centrifuge (1,000 r/min, 1 min) to remove serum and buffy coat. Then, RBCs were resuspended and diluted by PBS into appropriate cell density. 200 µL erythrocyte suspension was dropped on the clean silanized slide to attach for 20 min at room temperature, the unabsorbed RBCs were washed away by PBS. For the live cell imaging, the cells were blocked by 3%BSA, then stained by Glu-probes in dark for 3 min at room temperature, or stained by antibody-probes in dark for 10 min at 4°C, or stained by Tyr-probe (or in presence of free Phe with its 300:1 ration to probe for the AA blocking experiment) in dark for 10 min at room temperature; then, excess probes were removed away by PBS washing for five times for the next normal dSTORM imaging experiment. For glucose-replacement experiment, 200 µL free glucose solution (5 mmol/L) was added and treated cells for 5 min at room temperature after normal staining cells with Glu-Alexa532. Next, cells were washed five times with PBS for dSTORM imaging experiment. For the fixed sample imaging, we applied post-fixation to ensure the normal cellular activity to perform glucose uptake when Glu-probe labeling. That is, after being stained well, cells were fixed with 4% Paraformaldehyde (PFA) at room temperature for 20 min. Then, the sample was washed five times with PBS for the next dSTORM imaging experiment. For dual-color dSTORM imaging, the sample was firstly labelled by Glu-Alexa532, then by antibody-Alexa647, with using the same protocol as that in single-color staining.

5.3 For dSTORM imaging of Hela cells

For the live cell imaging, the well cultured cells were washed with PBS and blocking by 3% BSA for 20 min at room temperature, then stained with the Glu-probes under an appropriate concentration for 3 min in dark at room temperature, or stained with the Tyr-probes for 10 min at room temperature. With dropping away the staining solution, the sample was washed five times with PBS. For the fixed cell imaging, after staining, cells were post-fixed with 4%PFA at room temperature for 20 min. The experiment methods in glucose-replacement experiment and AA-blocking experiment

was same as those for imaging of the RBCs.

For imaging the sample stained by Glu-probe and antibody-probe, to get rid of potential impact of the glucose oxidase and free glucose in the classical imaging buffer on the Glu-probe, we used the other one: the imaging buffer ¹ containing 10 nM protocatechuate 3,4-dioxygenase (PCD, Sigma-Aldrich), 2.5 mM protocatechuic acid (PCA, Sigma-Aldrich), and 1mM Trolox (Sigma-Aldrich); for imaging the samples stained by Tyr-probe, the imaging buffer was same as the classic formula. With dropping 50 μ L imaging buffer on a large microscope slide (24 mm \times 50 mm, Fisher), and the small cover slide (22 mm \times 22 mm, Fisher) where cells were cultured was gently sealed on the large one with nail polish.

6. Confocal microscopy imaging

Fluorescent images were collected by using an inverted Ti-E microscope (\times 100/1.49 oil objective) (Nikon, Japan) equipped with a spinning disk confocal imaging system (Andor, Oxford Instruments). Sample preparation was the same as that in dSTORM imaging.

7. dSTORM imaging

dSTORM imaging was performed by using an inverted Nikon Ti-E microscope with an oil-immersion 100 \times 1.49 NA lens (Nikon, Japan). To image cell apical membrane which is the medium exposed side, the sample was illuminated by adjusting the excitation inclination to maximize the signal-to noise ratio. For single-color imaging, the sample was excited with a 532 nm laser only. For dual-color imaging, the sample was first excited with a 640 nm laser for antibody-Alexa647 labeled imaging and then with a 532 nm laser for Glu-Alexa532 labeled imaging. The imaging sequence 8000 frames (512 \times 512 pixels, 130 nm/pixel) was acquired by *Micro-Manager* based on *ImageJ* (U.S. National Institutes of Health) with a high-sensitive EMCCD (electron-multiplying charge-coupled device, Photometrics, Cascade II) camera with an exposure time of 20 ms. During imaging (usually less than 5 min), we stabilized the sample with two clips to minimize the possibility of x-y drift and eliminated the z-drift with an auto-focus lock, thus the stage shift could be ignored. Moreover, the Drift was correct in the data post-processing by *ThunderStorm*.

8. Data analysis

We applied *ThunderStorm* based on *ImageJ* to analyze the raw data and gained a reconstructed dSTORM image. First, according to our experiment condition, we set the private camera parameters; then we chose the suitable analysis method and parameter thresholds to analyze the raw data and gained the localizations of the imaging sequence; with setting several important parameters (such as the sigma, the intensity, the offset,

the uncertainty) thresholds, we removed the “bad localization”; with applying the “merge” and “drift correct”, we finally reconstructed a dSTORM image at nanoscale resolution.

The localization density on the cellular membrane was calculated as previous article depicted ². In details, an intrinsic image process in *ImageJ* was exploited to measure the area of the cell membrane of interest, and then the accurate total number of points on the corresponding cell membrane was acquired by performing *MATLAB*. Finally, we obtained the point densities on the cellular membrane under the different labeling concentrations, and then plotted the labeling concentration curves for Glu-probe and antibody-probe.

Cluster analysis was implemented by *SR-Tesseler* method ³. In this analysis, a Voronoï diagram was used to subdivide a super-resolution image into polygons centered on localized molecules. Through comparing the localization distribution to a reference uniform distribution, the suitable thresholds could be inferred. With defining δ as average density on the image and δ^l_i as the first-rank density, the localizations can be automatically segmented by selecting and merging all the polygons having $\delta^l_i > \alpha\delta$, where α is a positive multiplicative coefficient. Then, the object segmentation process is computed with setting thresholds by either manually adjusting or automatically determining. For practical applications, after reconstruction of dSTORM image by loading coordinates of localizations, the cellular membrane of interest was selected to create polygons by performing the Voronoï diagram. With setting the density factor, cut distance, min area and min#locs, objects were created and their shape and outline were showed. Then, clusters can be further computed from the ‘cluster’ tab, with adjusting the density factor and min area and min#locs. Finally, cluster area, number of points in cluster, coordinates and morphological parameters (by principal component analysis) are computed and displayed.

Colocalization analysis was performed by the CBC method ⁴ in *ThouderStorm* to characterize the spatial association of localizations from Glu-Alexa532 labeling and antibody-Alexa647 labeling. With taking into account the spatial distribution of biomolecules, the CBC analysis provides a colocalization value for each single-molecule localization of each species separately. This method compares the spatial distribution of surrounding localizations from both species to correct for multiple detection events. For each molecule of a species A, the average number of molecules of a species B in a given distance is calculated. To overcome the false positive colocalization information for localizations where the nearest neighbor is far away, the colocalization is weighted by the distance to the nearest neighbor. As a result, the CBC value is calculated from single-molecule localization data of two species (A, B), and

C_{Ai} is assigned to each single localization (i.e., A_i) of each species (i.e., A). C_{Ai} can adapt values from -1 to 1. As C_A value increases from -1 to 1, the spatial relationship of points from two channels also became more and more closer, from anti-correlated ($C_A=-1$) through no colocalization ($C_A=0$) to perfectly correlated distribution ($C_A=1$). Finally, the distribution percentage of C_A larger than 0 could show the degree of colocalization of points from two channels.

References

1. C. E. Aitken, R. A. Marshall and J. D. Puglisi, *Biophys. J.*, 2008, **94**, 1826-1835.
2. J. Chen, J. Gao, J. Wu, M. Zhang, M. Cai, H. Xu, J. Jiang, Z. Tian and H. Wang, *Nanoscale*, 2015, **7**, 3373-3380.
3. F. Levet, E. Hosy, A. Kechkar, C. Butler, A. Beghin, D. Choquet and J. B. Sibarita, *Nat Methods*, 2015, **12**, 1065-1071.
4. S. Malkusch, U. Endesfelder, J. Mondry, M. Gelleri, P. J. Verveer and M. Heilemann, *Histochemistry and cell biology*, 2012, **137**, 1-10.