Electronic Supplementary Material (ESI) for Nanoscale.

Supporting information

Development of small molecule inhibitor-based fluorescent probes for high specific super-resolution imaging

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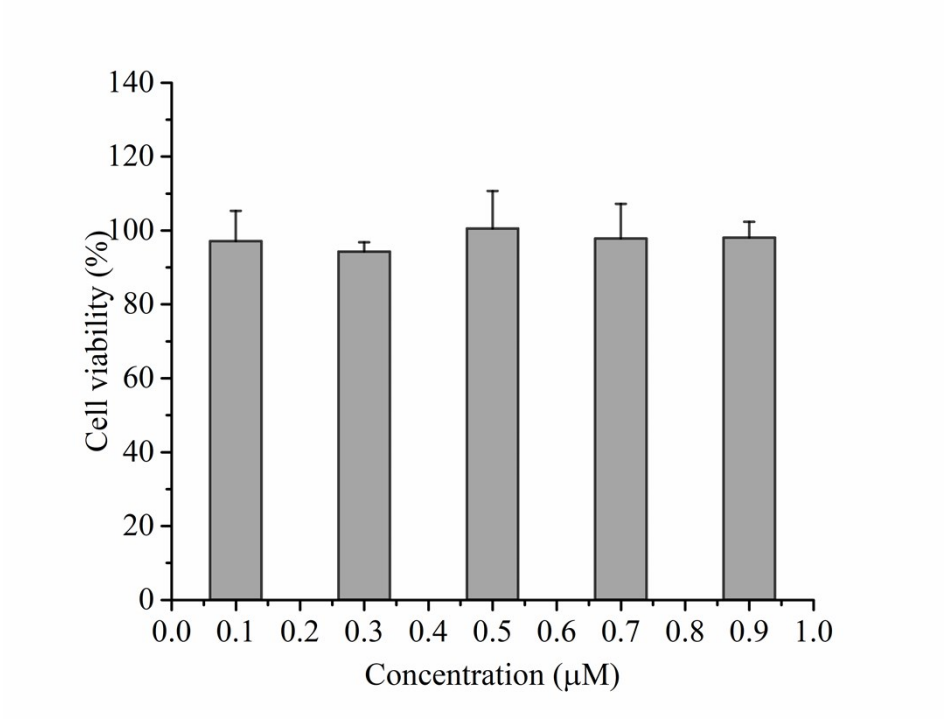


Figure S1. Detection of the toxicity of different concentrations of Gefitinib-probe to A549 cell with cell counting kit-8 (CCK-8).

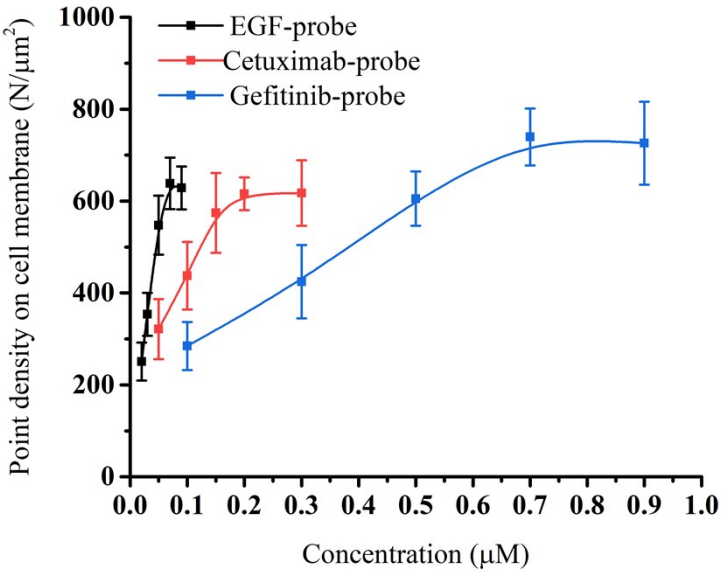


Figure S2. Concentration gradient curves of three fluorescent probes, including the EGF-probe, the Cetuximab-probe and Gefitinib-probe. Data of every labelling concentration were acquired from more than 10 cells in three independent experiments.

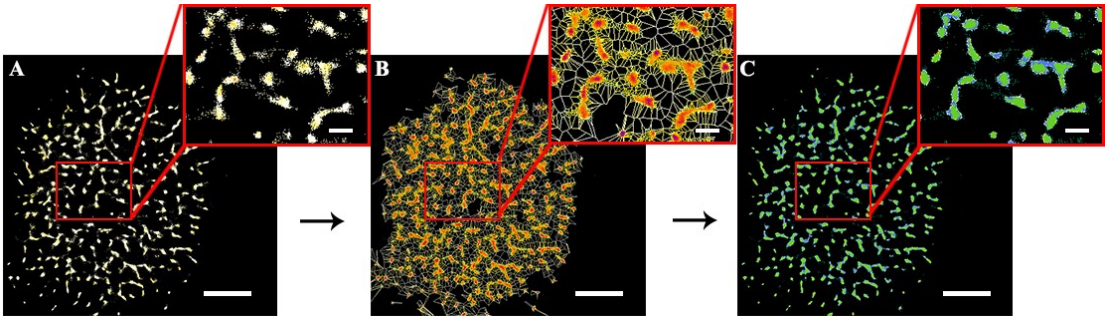


Figure S3. Graphical representation of the SR-Tesseler analysis process. (A) The original image rendered after the localization data was imported. (B) The Voronoï diagram was reconstructed by creating polygons based on local density. (C) The qualified clusters are abstracted from the segmented objects with setting density factor and cut distance. Scale bars in big images are 5 μm, and scale bars in the enlarged images are 1 μm.

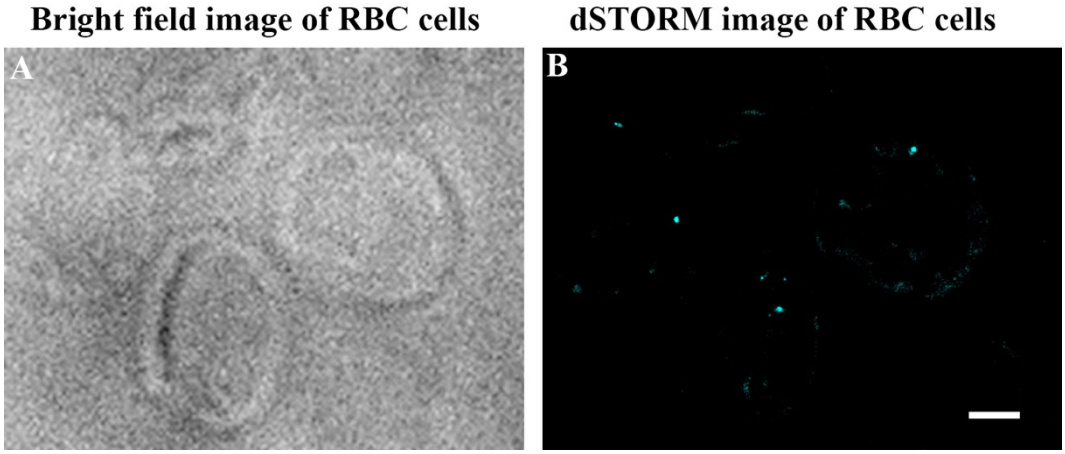
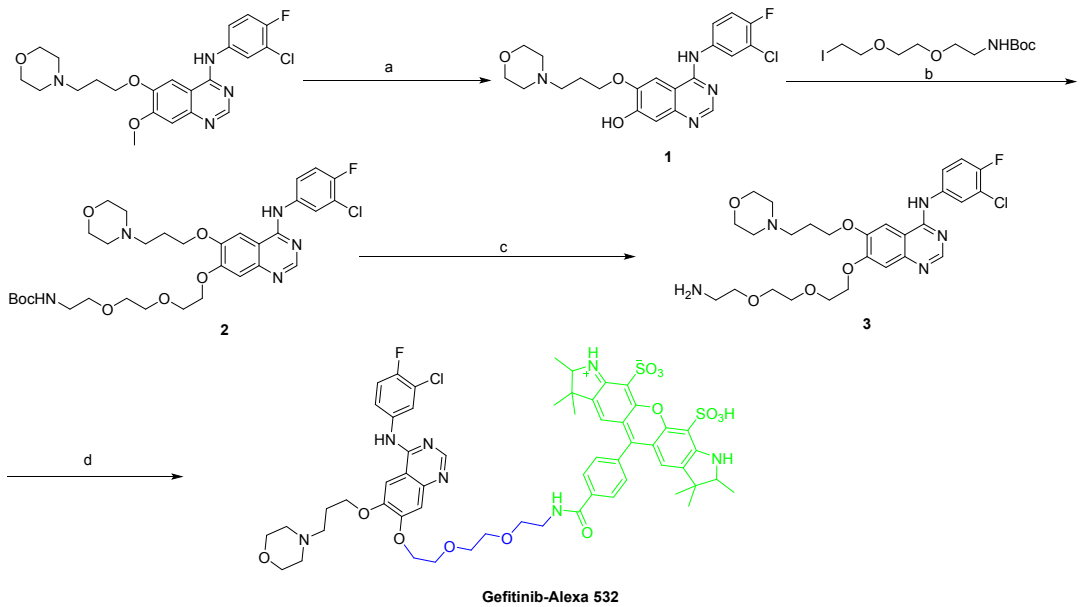


Figure S4. The Bright field image and reconstructed dSTORM image of RBC cells with Gefitinib-Alexa532 staining. Scale bar are 2 μm.

Experimental Methods

1. Synthesis of Gefitinib-probe



Scheme 1: Synthesis route of Gefitinib-Alexa532. Reagents and conditions: a) $AlCl_3$, DCM, 0°C to reflux, 16 h; b) PEG linker, K_2CO_3 (3.0 eq.), ACN, reflux, 3 h; c) TFA/DCM (v/v = 1:5), rt, 2 h; d) Alexa532, DIEA, 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 *d6*-DMSO. Tetramethylsilane (δ = 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.

Compound 1

To a stirred solution of Gefitinib (500 mg, 1.12 mmol) in DCM (60 mL) was added $AlCl_3$ (1.2 g, 8.96 mmol) at 0°C, then the resulting solution was heated to reflux for 16 h, cooled to 0°C, and saturated sodium bicarbonate was added. The suspension was separated and the organic layer was collected. The aqueous layer was extracted with DCM/MeOH (10:1) for three times. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give **compound 1** (80 mg, 17% yield) as a yellow solid. ^{1}H NMR (400 MHz, CDCl3): δ 8.62 (s, 1H), 7.95–7.93 (m, 1H), 7.58-7.56 (m, 1H), 7.49 (s, 1H), 7.31 (s, 1H), 7.15 (t, 1H, J = 8.74 Hz), 4.11 (t, 1H, J = 5.17 Hz), 3.83 (t, 4H, J = 4.61 Hz), 2.68 (t, 1H, J = 6.08 Hz), 2.63 (br, 4H), 2.06-2.01 (m, 2H).

Compound 2

To a stirred solution of compound 1 (45 mg, 0.1 mmol) in acetonitrile (1 mL) were added PEG linker (45 mg, 0.12 mmol) and K_2CO_3 (43 mg, 0.3 mmol), then the resulting solution was heated to 50°C for 10 h. Cooled to room temperature, the mixture was diluted with ethyl acetate, then washed with water and extracted with EA for three times. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography with eluent (DCM/MeOH = 10:1, v/v) to afford compound 2 (40 mg, 60% yield) as a light yellow oil. ^{1}H NMR (400 MHz, CDCl3): δ 8.64 (s, 1H), 7.95–7.93 (m, 1H), 7.60-7.58 (m, 2H), 7.26 (s, 1H), 7.16 (t, 1H, J = 8.8 Hz), 5.12 (s, 1H), 4.31 (t, 2H, J = 5.00 Hz), 4.20 (t, 2H, J = 6.33 Hz), 3.95 (t, 2H, J = 4.33 Hz), 3.80 (t, 4H, J = 5.17 Hz), 3.75 (t, 2H, J = 4.98 Hz), 3.64 (t, 2H, J = 3.88 Hz), 3.53 (t, 2H, J = 4.90 Hz), 3.30 (m, 2H), 2.68 (t, 1 H, J = 7.10 Hz), 2.60 (br, 4H), 2.14-2.09 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, CDCl3): δ 155.78, 155.68, 153.97, 152.92, 148.67, 140.91, 135.04, 123.66, 121.31, 121.25, 116.19, 115.97, 108.70, 108.52, 101.12, 70.52, 69.93, 68.86, 68.15, 67.21, 66.20, 54.99, 53.21, 39.99, 28.00, 25.47. HRMS: calcd. for $C_{32}H_{42}ClFN_5O_7$ $[M-H]^-$ 662.27623; found 662.27726.

Compound 3

To a stirred solution of compound 2 (40 mg, 0.06 mmol) in DCM (1 mL) was added TFA (0.2 mL), then the mixture was stirred at room temperature for 2 h. Concentrated under reduced pressure to afford the compound 3 (45 mg) as crude product without purification for next step.

Gefitinib-Alexa 532

For the conjugation, 85 μg Alexa532 was dissolved in 10 μL dry DMF and add to 90 μL compound **3** diluted solution (0.2 mg/mL, in dry DMF), then mixed thoroughly. The resulting solution was reacted in the dark at room temperature overnight with constant shaking. The DMF was evaporated under reduced pressure, the residue was purified by illustra NAP-5 columns (GE Healthcare) with phate-buffered isotonic saline

(PBS) as eluent to afford the desired Gefitinib-Alexa532. 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) was collected for labeling experiment.

2. Linkage of Cetuximab to organic dye

The commercial Cetuximab was diluted to the appropriate concentration with PBS (commonly, 100 μg/mL, 100μL), then 0.2 μL Alexa532 (or Alexa647) (1mg/mL, Invitrogen) was added to react with Cetuximab in the dark at room temperature with continuous vortex for 2.5 h. After reaction, the excessive free dyes were removed by separation with illustra NAP-5 columns (GE Healthcare). The combination ratio of dye to Cetuximab was determined by UV-visible absorption spectroscopy assay. Finally, the qualified solution with a suitable ratio (0.8-1.0) was collected for labeling experiment. EGF-Alexa532 was prepared by a similar method as described above.

3. Cell culture

A549 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 μg/mL penicillin and 100 μg/mL streptomycin, in humidified incubator at 37℃ (with 5% CO2). 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.

4. Sample preparation

For dSTOMR imaging of EGFR

Well cultured A549 cells were washed with PBS for three times and fixed with 4% Paraformaldehyde (PFA) at room temperature for 40 min. Then, the cells were washed with PBS for three times and blocked by 3% Bovine Serum Albumin (BSA) at room temperature for 20 min. Last, the sample was washed with PBS for three times again and stained with labeling probe (Gefitinib-probe, EGF-probe, Cetuximab-probe) in the dark for 10 min at 4°C. Excess probes were removed away by PBS washing for 4-5 times for the normal dSTORM imaging experiment.

For dual-color dSTORM imaging of EGFR

Well cultured A549 cells were fixed, blocked and stained with EGF-Alexa647 (Invitrogen) as the methods in the single-color dSTORM imaging of EGFR, then the cells were washed for 5 times and stained with Gefitinib-Alexa532 in the dark for 10 min at 4°C. Excess probes were removed away by PBS washing for 4-5 times for the dual-color dSTORM imaging experiment.

For dual-color dSTORM imaging of EGFR and HER3

The well cultured cells were washed 3 times with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature for 40 min. The cells were then washed 3 times and blocked at 3% BSA for 20 min at room temperature. 1B4C3 monoclonal antibody as HER3antibody (Biolegend) (1 μg/mL, in 1% BSA) was added to the cells and incubated overnight at 4°C. After washing 3 times with PBS for 5 min each, cells were stained with anti-mouse IgG-Alexa647 (1 μg/mL in 1% BSA; Invitrogen, 1857666) in the dark at room temperature for 1 h, washed 4 times with PBS for 3 min each. Next, the sample was stained with Gefitinib-Alexa532 (0.7 μM) or Cetuximab-Alexa532 (0.2 μM) in the dark at 4°C for 10 min. Finally, cells were washed with PBS for 5 times to remove excess dye for dual-color dSTORM imaging.

5. dSTORM imaging

Drop 50 μL of imaging buffer on a large glass slide (24 mm × 50 mm), the imaging buffer contains: Tris (50 mM, PH 8.0), NaCl (10 mM), glucose (10% w/v), glucose oxidase (500 μg/mL, sigma), catalase (40 μg/mL, sigma), and β-mercaptoethanol (β-ME, 1% v/v, sigma). The small coverslip (22 mm × 22 mm) containing cells was slowly poured over the lager slide and sealed with nail polish.

dSTORM imaging experiment was accomplished by an inverted Nikon Ti-E microscope with an oil-immersion 100×1.49 NA lens (Nikon, Japan). To image cellular apical membrane under the lowest background, 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 HER3antibody or EGF-Alexa647 labeled imaging and then with a 532 nm laser for Cetuximab-Alexa532 or Gefitinib-Alxea532 labeled imaging for EGFR. The high sensitive EMCCD (electronic multiplier charge coupled device, photometry, cascade II) camera was used to acquire 5000 images at high-speed frame (512 × 512 pixels, 130 nm per pixel) at 20 ms exposure time by combining with Micro-Manager based on ImageJ (U.S. National Institutes of Health). During imaging, Nikon micro imaging equipment provides a perfect focus system (PFS) to realize the real-time correction of the focus drift in the y axis, and four-color particle microsphere (100 nm, Invitrogen) was used as the benchmark to correct the X-Y drift.

6. Data analysis

The raw data was analyzed by ThunderSTORM based on ImageJ to yield qualified localizations and reconstruct dSTORM image. Firstly, the private camera parameters were set according to our experiment conditions; then, the appropriate analysis method and parameter thresholds were selected to analyze the raw data and achieve the localizations of the imaging sequence; with setting the vital parameters (such as the

sigma, the intensity, the offset, the uncertainty) thresholds, the qualified localizations were obtained with removing the “bad localization”; after 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 reported.[1] In detail, we used the inherent image process in ImageJ to measure the area of the cell membrane of interest and exploited the MatLab to acquire the total number of points on the cell membrane of interest. Then, the point density on the cell membrane was obtained. By calculating the point density values on the cell membrane at different labeling concentrations of the probe, we plotted the gradient curve of the labeling concentration of the fluorescent probe.

Cluster analysis was performed by SR-Tesseler.[2] SR-Tesseler is based on Voronoï diagrams and able to precisely and automatically segment protein clusters by using a local density parameter and finally characterize protein organization at different scales. In the case of SMLM data, the Voronoï diagram is built from molecule coordinates as the seeds, with the super-resolution image being subdivided into polygons centered on each seed. Each polygon Pi can be considered as the influence region of the corresponding seed si, and its geometric characteristics such as polygon area Ai or shape can describe the neighborhood of the seeds. First-rank neighboring polygons P1i,j of si are defined as the n1i polygons of area A1i,j sharing edges with Pi, centered on the seeds s1i,j. Similarly, higher rank polygons Pki,j, $k > 1$, are defined as the nki polygons sharing edges with Pk−1i,j. The neighborhood of si at the kth rank is then defined by the {n1i, …, nki} localized molecules. For each seed si, various parameters can be computed, such as the area Aki, the density δki, the mean distance dki, the shortest distance (i.e., distance to the closest seed) Δki and the shape index ρki (Online Methods). These parameters provide quantitative information on the localizations surrounding each seed. With the Voronoï diagram being reconstructed, the parameters are computed, and the object segmentation process then is computed. With defining δ as average density on the image and δ1i as the first-rank density, the localizations can be automatically segmented by selecting and merging all the polygons having $\delta 1i > \alpha\delta$, 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 on our data, after reconstruction of dSTORM image by importing coordinates of localizations, the region of interest was selected to create the Voronoï diagram. With setting the density factor, cut distance, min area and min#locs, objects were recognized and their shape and outline were showed. Then, clusters can be further abstracted from the objects, with setting the density factor and min area and min#locs. Finally, cluster area, number of points in cluster, and morphological parameters (by principal component analysis) are computed and displayed.

Colocalization analysis was performed by the coordinate-based colocalization (CBC) method[3] in ThouderStorm to characterize the spatial association of localizations from two imaging channels. In this analysis, each location of the two species (A, B) is assigned a CBC value. To calculate the CBC value for a specific location Ai from species A, the distribution of locations from both species A and B

around Ai is calculated. Then, the colocalization value, CAi calculated for every single molecule location and can adapt values from -1 through 0 to 1, which can be used to describe the spatial relationship of two species, from anti-correlated (CA = -1) through no colocalization (CA = 0) to perfectly correlated distribution (CA = 1). In detailed analysis, after respectively importing the colocalizations from two channels into ThouderStorm to reconstruct the dSTORM images, the "CBC" was applied to calculate the colocalization values CA. As CA value increases from -1 to 1, the spatial relationship of points from two channels also became closer and closer, from anti-correlated (CA = 1) to perfectly colocalized distribution (CA = 1). Finally, the percentage of CA greater than 0 can be used to quantify the degree of co-localization of points from two probes labeling.

References

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[2] F. Levet, E. Hosy, A. Kechkar, C. Butler, A. Beghin, D. Choquet, J. B. Sibarita, *Nat. Methods* **2015**, *12*, 1065.

[3] S. Malkusch, U. Endesfelder, J. Mondry, M. Gelleri, P. J. Verveer, M. Heilemann, *Histochem. Cell Biol.* **2012**, *137*, 1.