Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Mechanistic Insights into EGFR Membrane Clustering Revealed by Super-resolution Imaging

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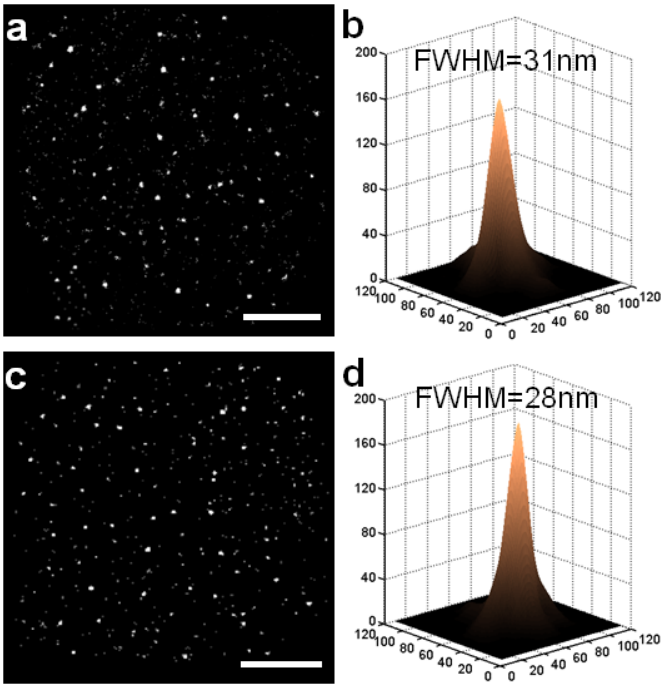

Fig. S1 Measurement of the localization precision of single Alexa647 conjugated Cetuximab or EGF in the membranes of living COS-7 cells. (a) A representative dSTORM image of adequately diluted Alexa647 conjugated Cetuximab molecules (≈10 nM) on APTES modified coverslip. (b) Aligned two-dimensional distribution of localizations from 100 individual fluorescent molecules in (a) obtained a mean localization precision of 31 nm by measuring the full-width at half-maximum (FWHM). (c-d) Measurement of the localization precision of Alexa647 conjugated EGF molecules relatively to (a) and (b). The FWHM of Alexa647-EGF is 28 nm. Scale bars indicate 2 μm.

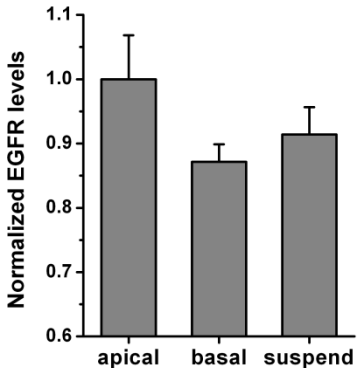

Fig. S2 Measurement of EGFR levels on different membrane surfaces of COS-7 cells. EGFR levels are calculated via dividing the total fluorescent localizations from individual cells by their individual surface areas. The level from the apical surface is set as one. The histograms shown are the means ± standard deviation (s.d.), which are obtained from 10 cell samples in 5 independent experiments.

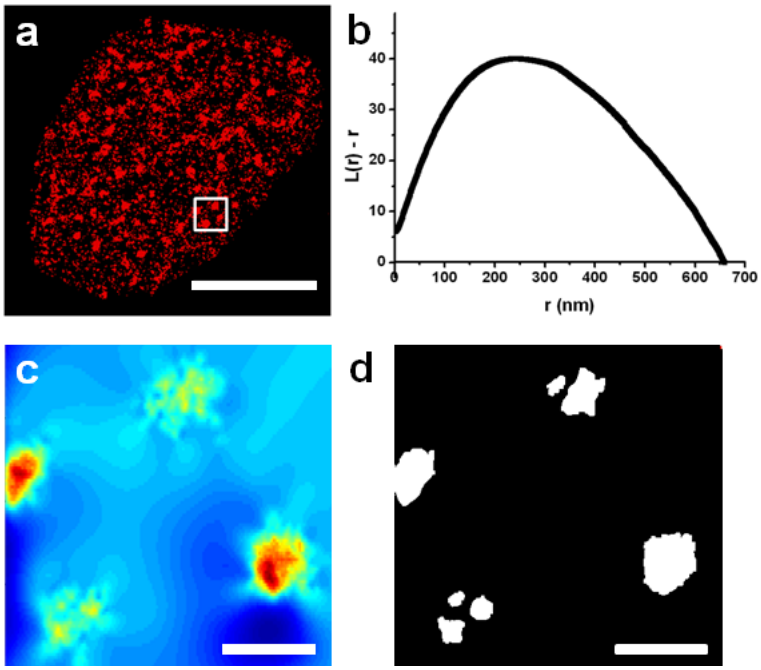

Fig. S3 Reconstructed dSTORM imaging of EGFR distribution and analysis the degree of clustering by Ripley's K function. (a) A typical reconstructed image of active EGFRs on the apical surface of the adherent COS-7 cell. Cells were labeled with Alexa647-conjugated Cetuximab. (b) Ripley's K function analysis of EGFR clustering in the 2×2 μm^2 region boxed in (a). The plot of L(r)-r showing clustering scales at 240 nm and the degree of clustering on length scales at 650 nm. (c) Interpolated cluster map boxed in (a) through Ripley's K function analysis indicating a highly clustering distribution. (d) Binary cluster image generated from the color-coded cluster map through a threshold of a defined L(r). Scale bars indicate 10 μm in (a), 500 nm in (c) and (d).

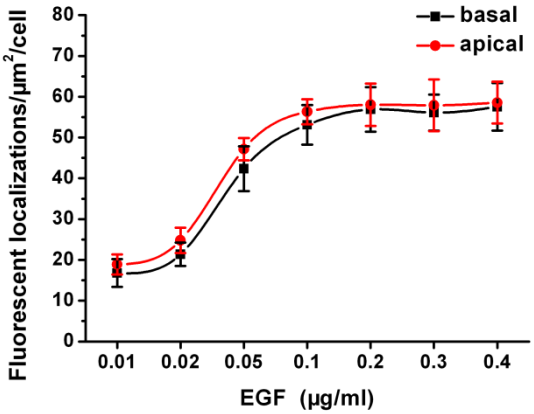

Fig. S4 Optimization of EGF labeling efficiency. In order to ensure the ligand binding as completely as possible and keep the endocytosis to a minimum, the number of fluorescent localizations per μm^2 on the apical and basal surfaces of COS-7 cells were tested under a series of different concentration of Alexa647-conjugated EGF. The plots show that localization numbers from both apical and basal surfaces almost reach the maximums when adding 0.1 μg/ml EGF to label the samples.

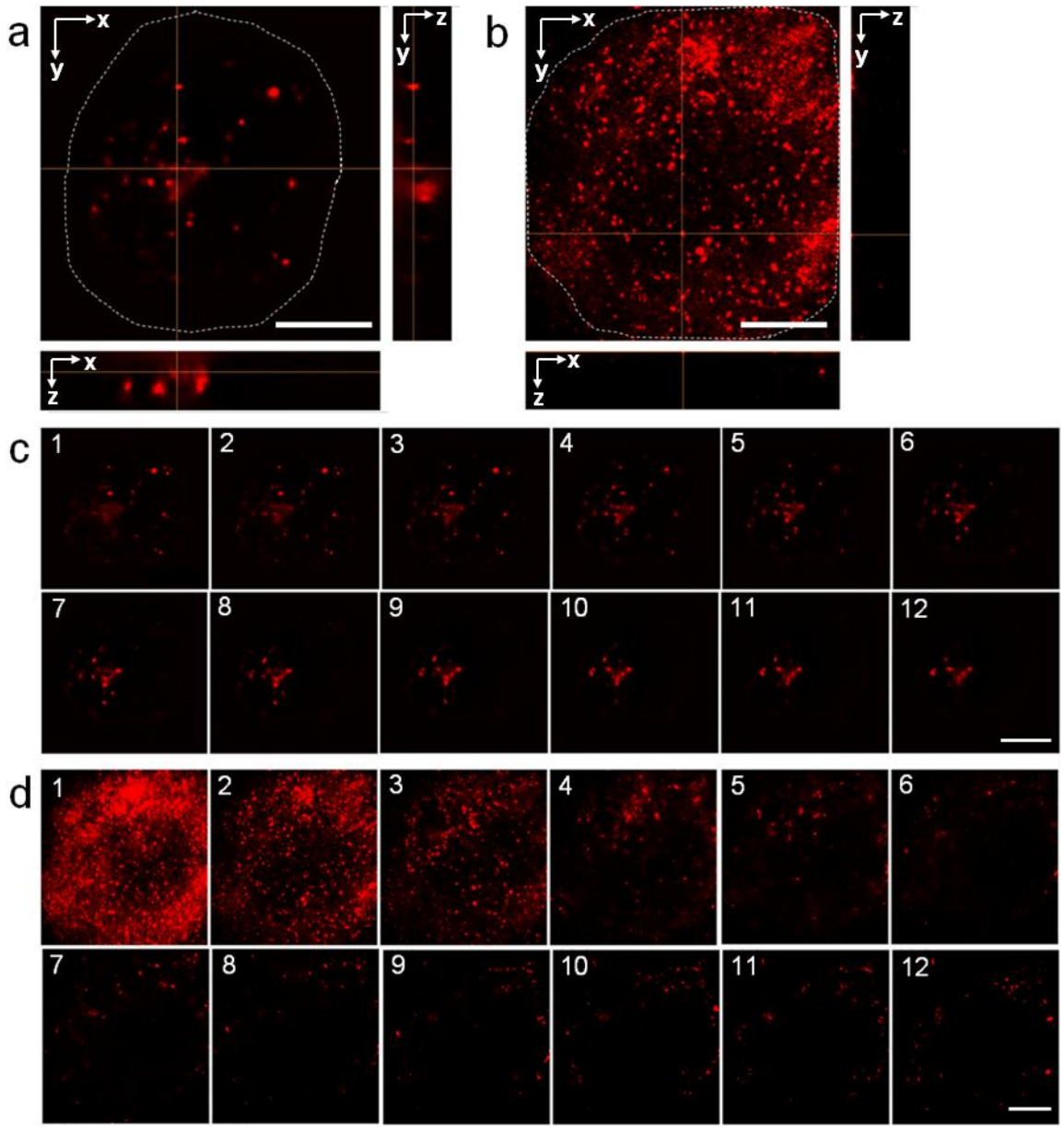

Fig. S5 Three dimensional fluorescent images of COS-7 cells stained by Alexa647-conjugated EGF. (a) Cells were incubated in no phenol red DMEM containing 0.1 μg/ml Alexa647- conjugated EGF at 37℃ for 20 min and imaged. To construct the 3D image, successive z-stacks spaced by 200 nm were recorded. The x-y image plane is located approximately 3 μm above the bottom of the cell and the cell outline is drawn by the dashed lines. The x-z and y-z image planes used for recording in all experiments are about 6 μm thick as indicated by the cross lines. (b) Cells were treated with 80 μM

dynasore at 37℃ for 30 min to inhibit EGFR endocytosis, washed by PBS containing 1% BSA for 3 times, and stained by 0.1 μg/ml Alexa647- conjugated EGF at 37℃ for 5 min. (c) Cells were treated as (a) mentioned. The images are a part of the successive z-stacks from the bottom to the top of the cell, showing the significant EGFR endocytosis. (d) Cells were treated as (b) mentioned. The images show that EGFR proteins are located in the membrane and hardly enter into the cell. Scale bars indicate 10 μm.

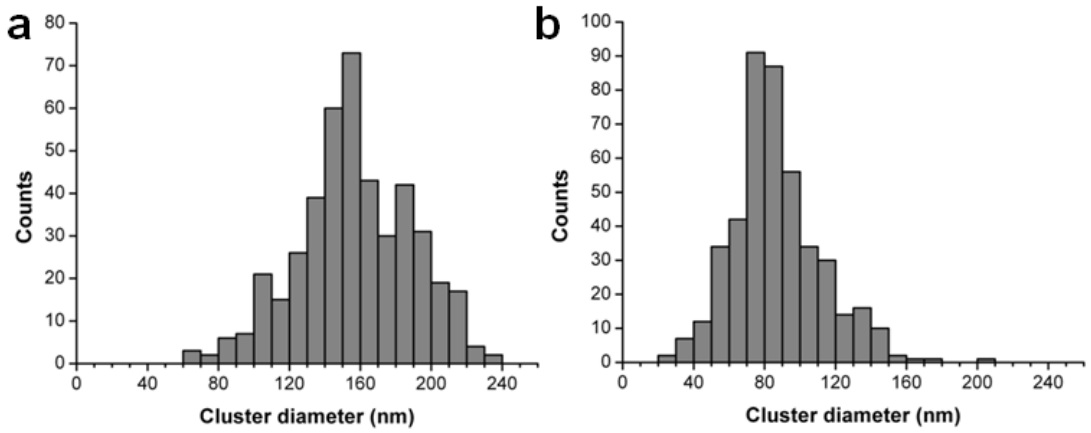

Fig. S6 Histograms of the diameters of lipid rafts. The diameter distribution of lipid rafts on the apical surfaces (a, n=4) and basal surfaces (b, n=4) of adherent COS-7 cells. The average diameter of clusters is 155±30 nm (s.d.) on the apical surfaces and 80±20 nm (s.d.) on the basal surfaces.

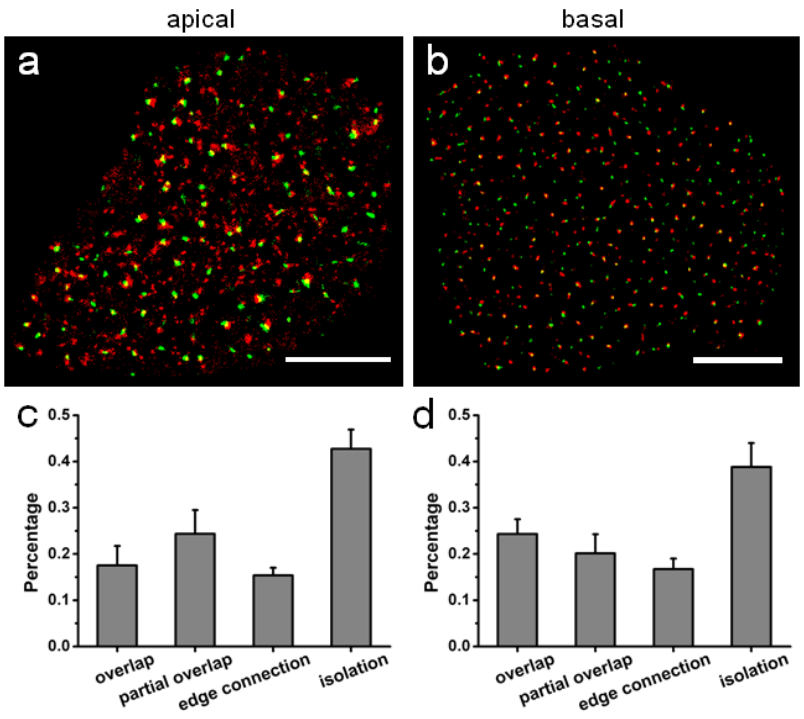

Fig. S7 Dual-color dSTORM imaging of inactive EGFRs and lipid rafts on fixed cell membranes. (a-b) The merging of EGFR (red) and lipid raft (green) channels show the significant colocalization of the two types of clusters on the apical (a) and basal (b) surfaces of fixed COS-7 cells. Cells were fixed with 4% paraformaldehyde in dark for 10 min and stained with Alexa647-conjugated Cetuximab at 4℃ for 10 min. Scale bars indicate 10 μm. (c-d) The distribution of EGFR and lipid raft spatial association on the apical (c) and basal (d) surfaces of fixed COS-7 cells. 600 cluster pairs from 5 independent cells are analyzed. Data are the means ± standard deviation (s.d.).

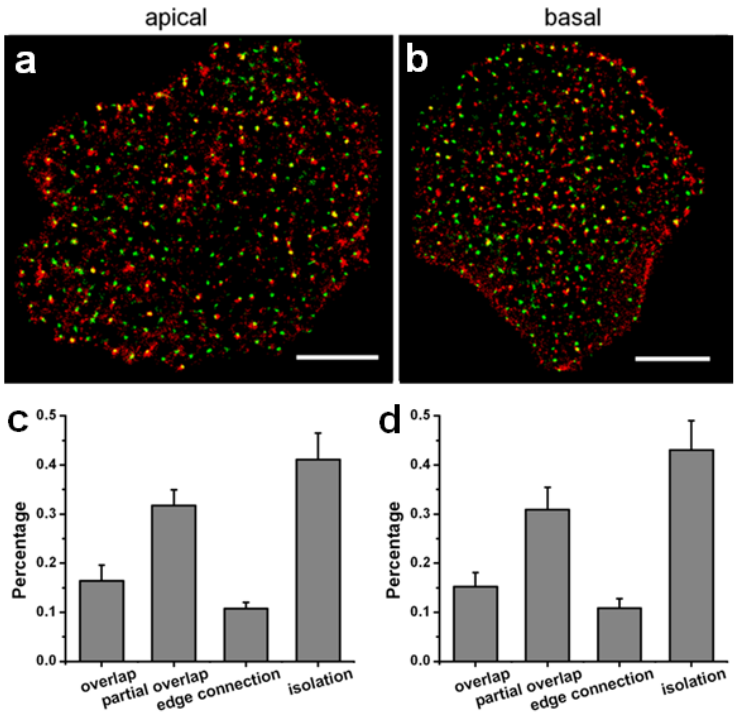

Fig. S8 Dual-color dSTORM imaging of active EGFRs and lipid rafts on living cell membranes. (a-b) The merging of EGFR (red) and lipid raft (green) channels show the significant colocalization of the two kinds of clusters on the apical (a) and basal (b) surfaces of adherent COS-7 cells. Cells were stimulated with Alexa647-conjugated EGF at 37℃ for 5 min and then kept on ice for inhibiting EGFR endocytosis during imaging. Scale bars indicate 10 μm. (c-d) The distribution of EGFR and lipid raft spatial association on the apical (c) and basal (d) surfaces of COS-7 cells. 600 cluster pairs from 5 independent cells are analyzed. Data are the means ± standard deviation (s.d.).

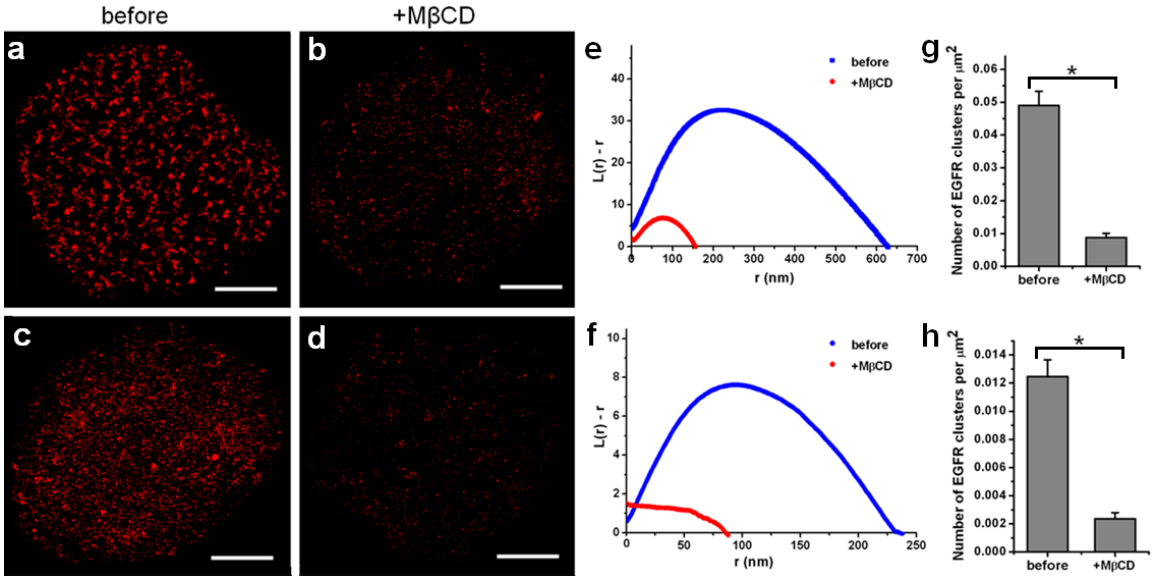

Fig. S9 Changes in the distribution of active EGFRs on the apical and basal membrane surfaces of COS-7 cells after treating with MβCD. (a-b) Reconstructed dSTORM images of active EGFRs on the apical surface of the adherent COS-7 cell before (a) and after (b) treating with MβCD. (c-d) Changes of the distribution of active EGFRs on the basal membrane surface as (a) and (b). Cells were stimulated with Alexa647-conjugated EGF and kept on ice during the imaging as control, and then added MβCD. Scale bars indicate 10 μm (a-d). (e-f) Representative Ripley's K function analysis of EGFR clustering on the apical (e) and basal (f) membrane surfaces before and after treating with MβCD. (g-h) Normalized number of EGFR clusters per μm^2 before and after adding MβCD on the apical (g) and basal (h) membrane surfaces. Data are the means ± standard deviation (s.d.), which were obtained from 10 cell samples in 5 independent experiments. * $p<0.05$, analysis of variance by two-tailed unpaired t-test.