Supplemental Information to
Reversible cryo-arrest for imaging molecules in living cells at high spatial resolution

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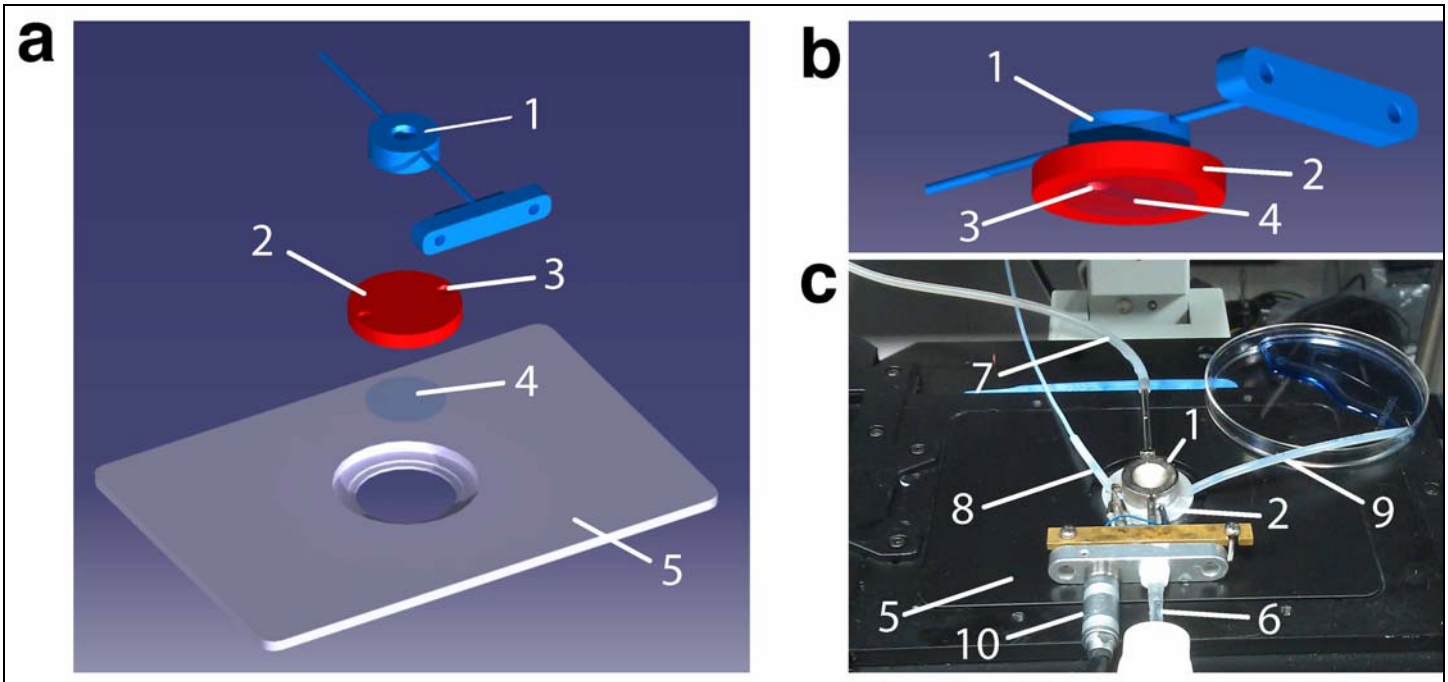
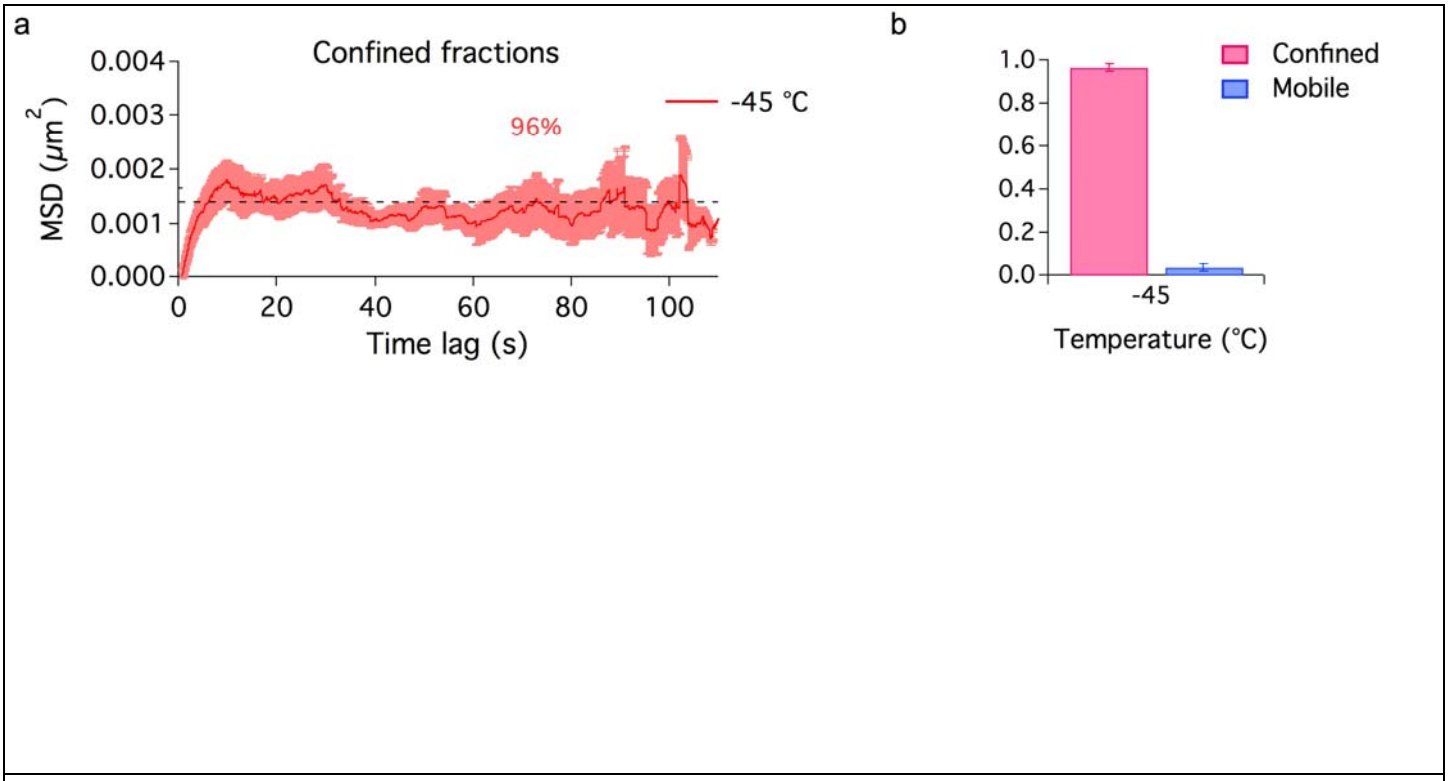


Supplementary Figure 1

Supplementary Figure 1: Design of the cryo-stage.

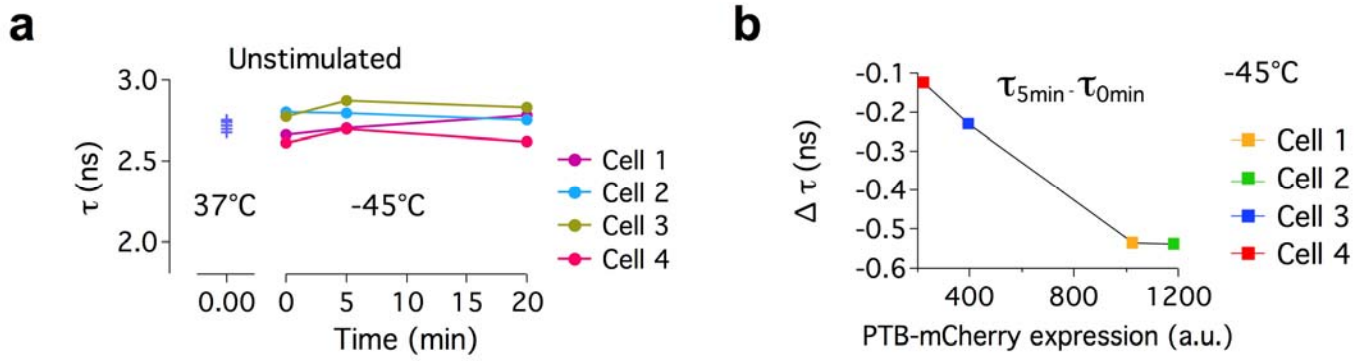
Shown are different representations of the assembly of the cryo-stage. **(a)** Computer-aided design (CAD) model of separated core parts of the cryo-stage. **(b)** CAD model of assembled stage without mounting viewed from below. **(c)** Photographic representation of the stage mounted on a microscope. (1) silver block with temperature control; (2) aluminum block with medium inlet and outlet (3) and 100 µm thick channel with a cover slide (4); (5) insulating polyvinyl chloride plate adapted for commercial microscopy stages; (6) nitrogen inlet connected to liquid nitrogen reservoir; (7) nitrogen outlet connected to nitrogen pump; (8) medium inlet connected to automated syringe; (9) medium outlet; (10) connection for electronic temperature measurement and control.



Supplementary Figure 2

Single particle tracking of Quantum Dots bound to EGF at -45°C.

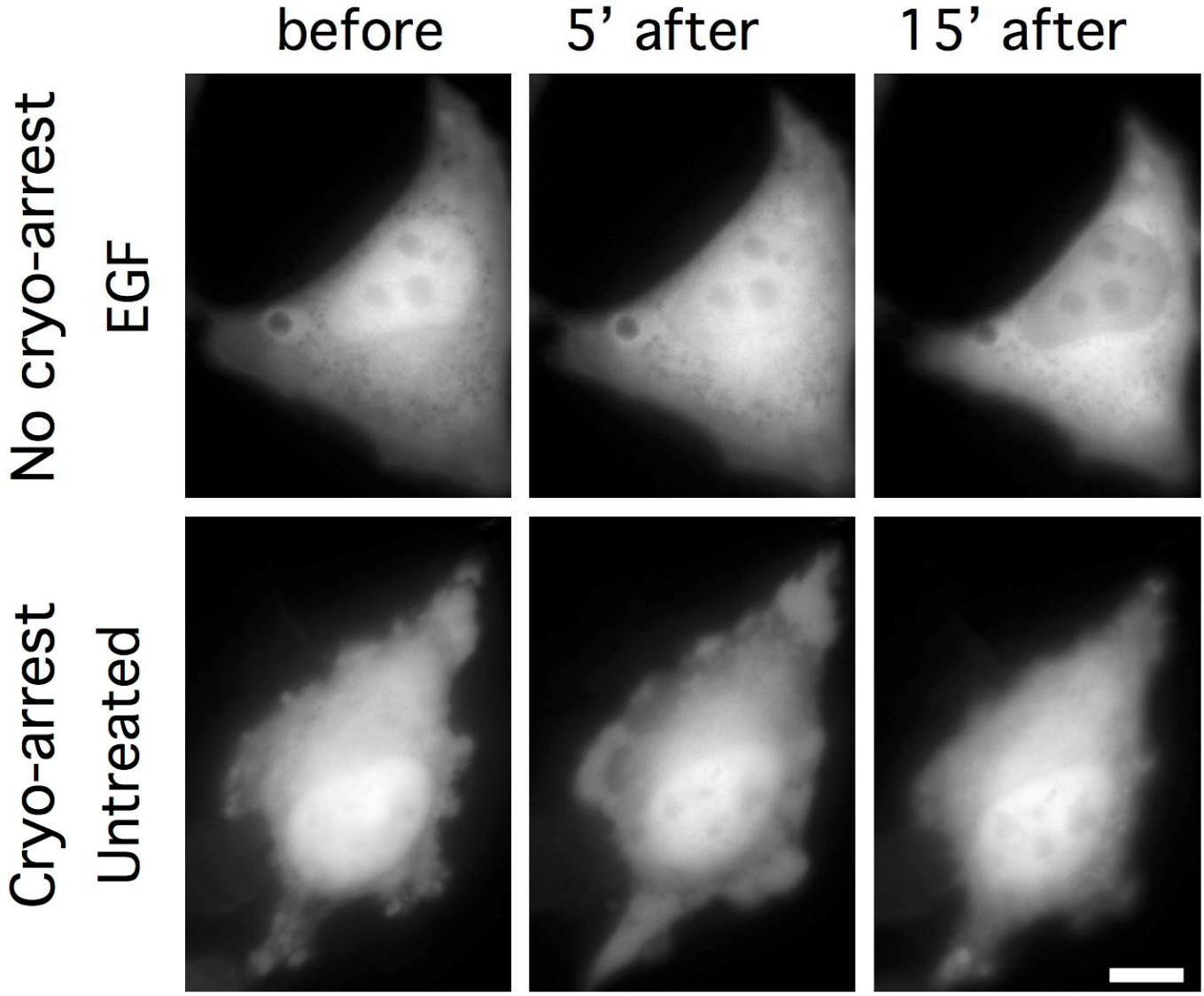
HeLa cells transfected with EGFR-mCitrine were incubated with QDots bound to EGF for 5 min and cryo-arrested. (n=5 cells) Single QDots were tracked over 2 min. (**a**) Mean squared displacement (MSD) of the confined fraction of tracks. Dashed line represents the MSD value related to the localization precision. (b) Fractions of detected molecule tracks that are classified as mobile and confined.



Supplementary Figure 3

Activation of EGFR signaling monitored under cryo-arrest.

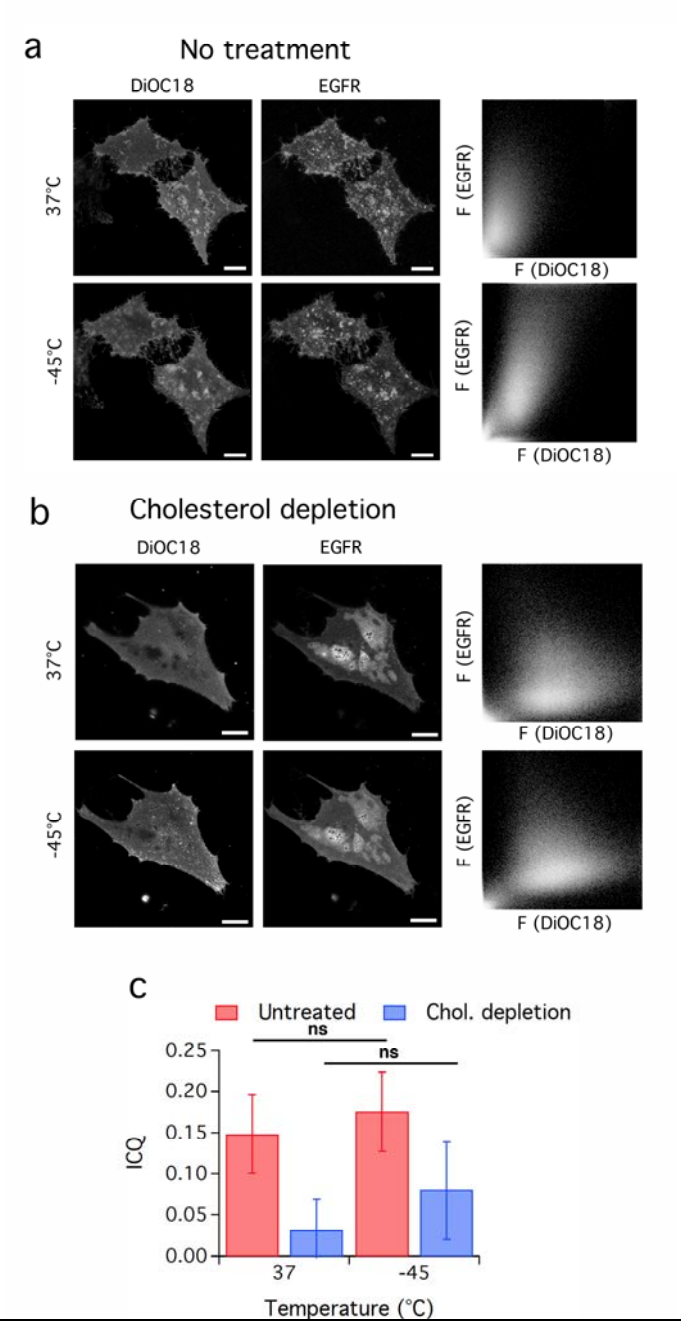
FRET-FLIM in living cryo-arrested HeLa cells co-expressing EGFR-mCitrine and PTB-mCherry. Cells were cryo-arrested in three consecutive cycles. (**a**) Average fluorescence lifetime (τ), of EGFR-mCitrine in four different cells cryo-arrested three times without stimulation compared to average fluorescence lifetime (τ) of EGFR-mCitrine at 37°C (4 cells). (**b**) Difference in average fluorescence lifetime ($\Delta\tau$) of EGFR-mCtrine in cryo-arrested HeLa cells before and 5 min after stimulation with 200 ng/mL EGF plotted against mean PTB-mCherry fluorescence intensity per cell.



Supplementary Figure 4

Localization of ERK-KTR upon stimulation with EGF and in untreated cells before and after cryo-arrest.

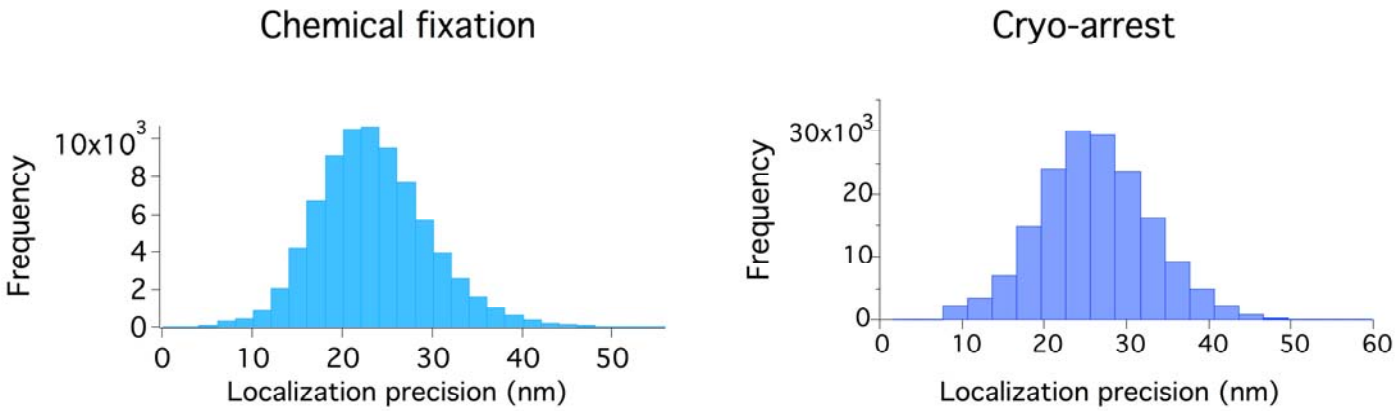
Representative images of HeLa cells at 37°C expressing ERK-KTR upon stimulation with 200 ng/mL EGF (upper row) or before and after a cryo-arrest cycle (lower row). Scale Bar: 10 μm



Supplementary Figure 5

Effects of cryo-arrest on EGFR partitioning in lipid domains.

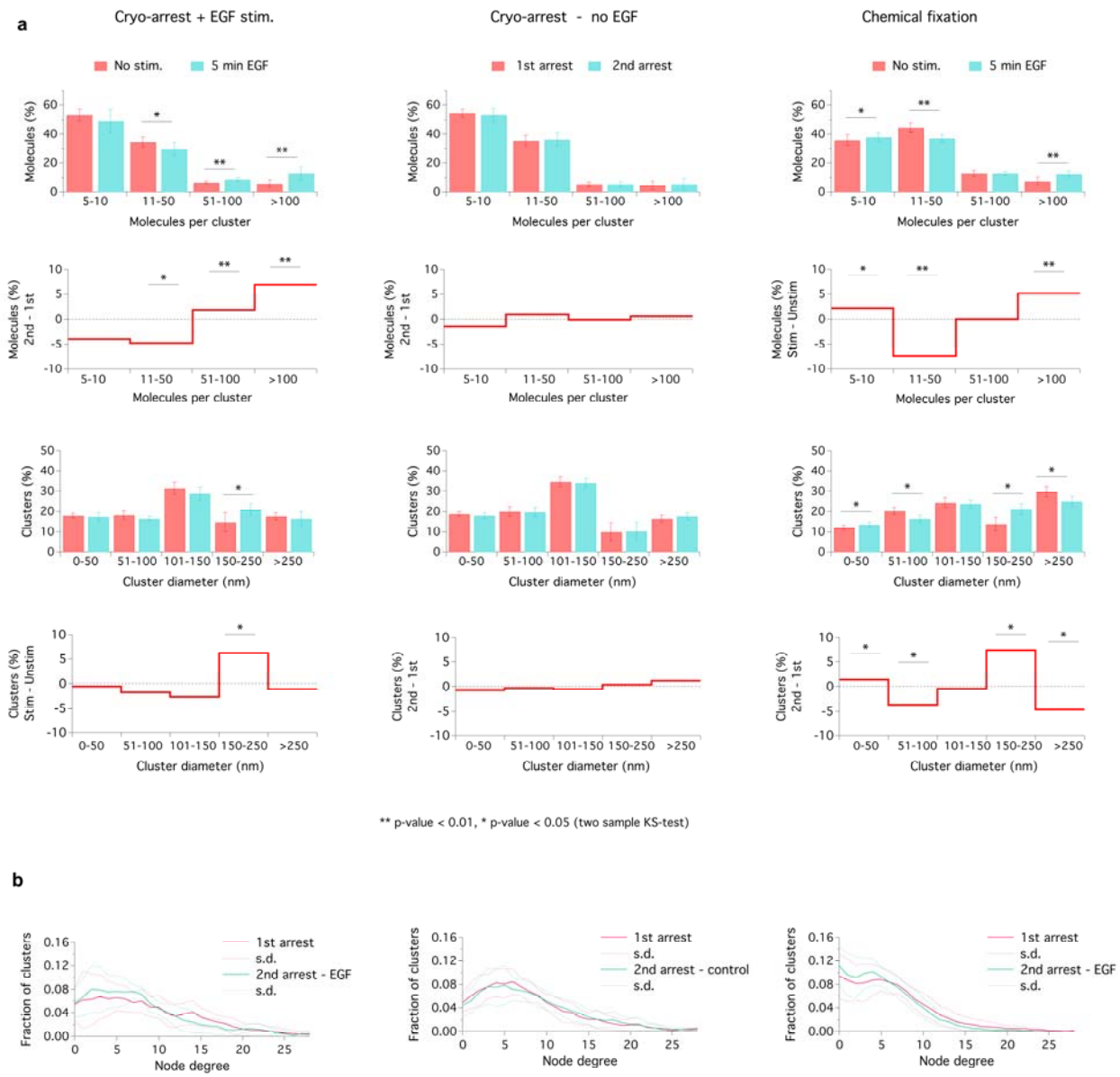
HeLa cells were transfected with a SNAP-EGFR construct, which was labeled with Alexa647 and the liquid-ordered domains in the plasma membrane were marked with the fluorescent dye DiOC18. **(a)** Representative confocal images of the basal membrane of untreated HeLa cells as well as scatterplots showing fluorescence intensity of Alexa-647 labeled EGFR versus DiOC18 fluorescence before (37°C, upper row) and during cryo-arrest (-45°C, lower row). **(b)** Representative images of HeLa cells, treated with 10 mM methyl-β-cyclodextrin for 60 min to extract cholesterol, before (37°C, upper row) and during cryo-arrest (lower row). **(c)** Quantification of co-localization of SNAP-EGFR with DiOC18 in HeLa cells with (n=12) and without cholesterol depletion (n=6) at 37°C and -45°C by image correlation quotient (ICQ); data is represented as mean ± s.d.; ns: $p > 0.05$ using student's t-test. Scale bars: 10 μm.



Supplementary Figure 6

Localization precision of EGFR-mEos2 obtained in PALM measurements.

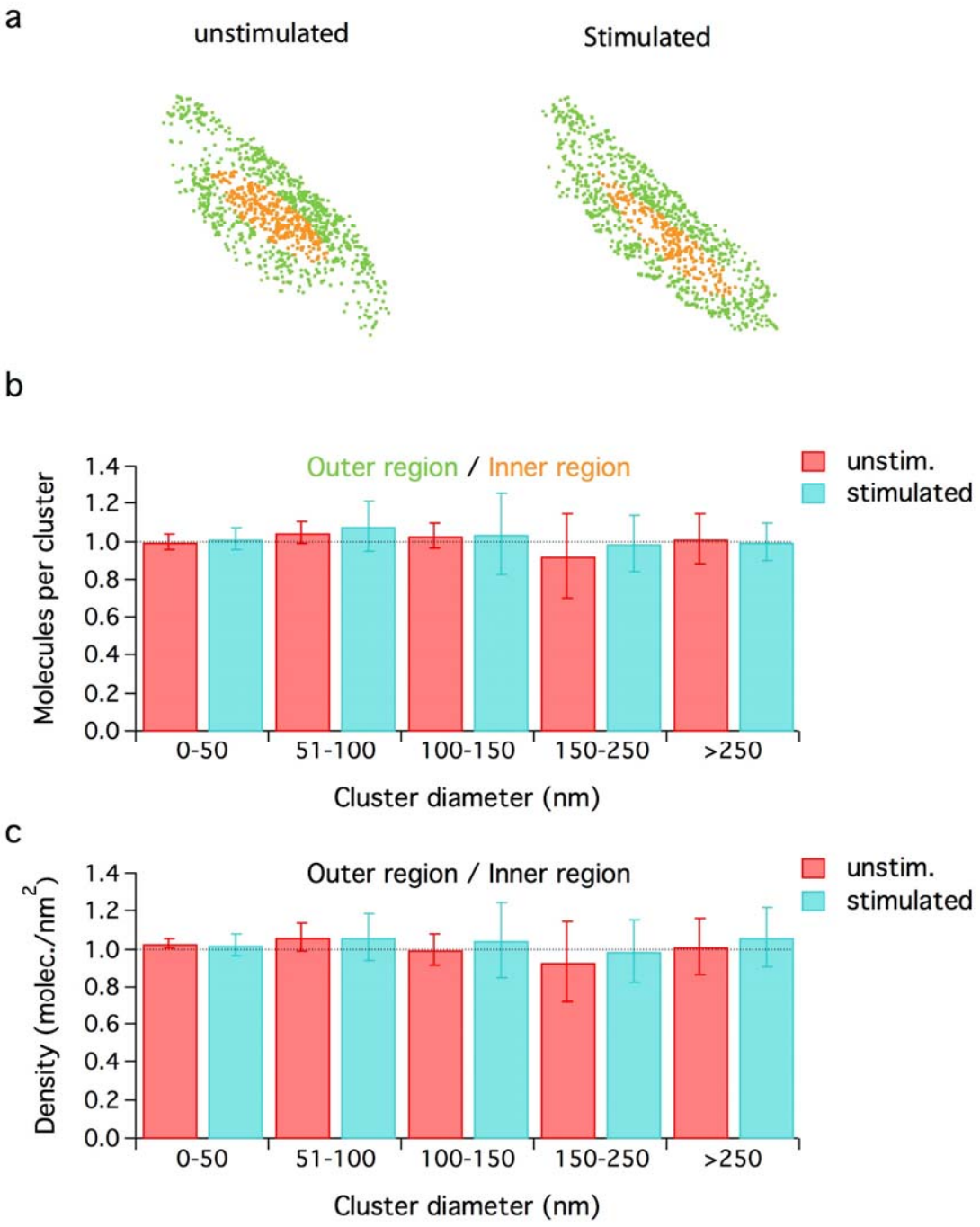
Localization precision histogram for EGFR-mEos2 molecules in HeLa cells that were chemically fixed by 4% formaldehyde (left) or cryo-arrested (right).



Supplementary Figure 7

Cluster analysis of EGFR in the basal plasma membrane.

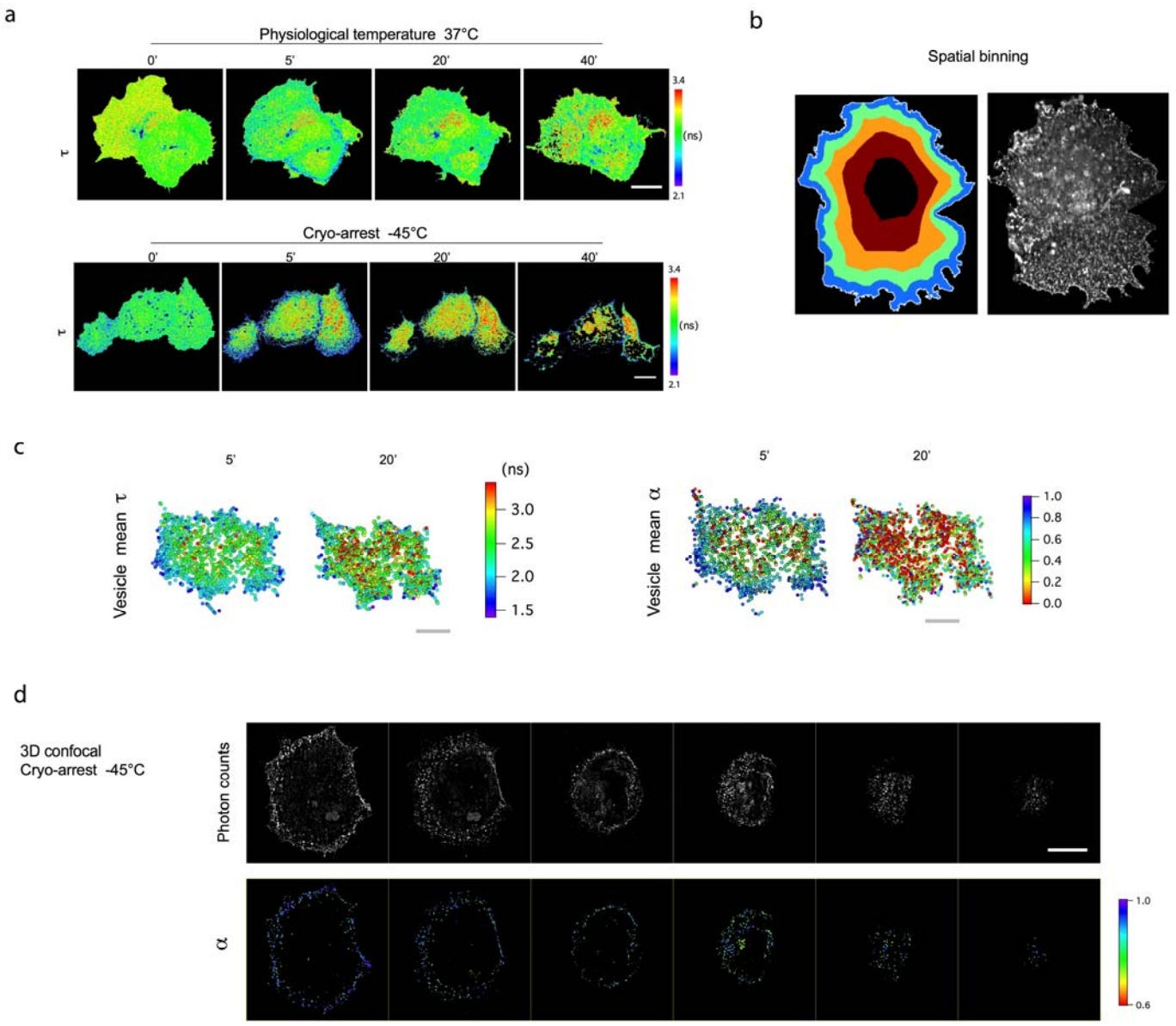
(**a**) Density-based spatial cluster analysis using a neighborhood radius of 40 nm of the EGFR-mEos2 distribution in the basal membrane of HeLa cells before (red) and 5 min after (blue) stimulation with EGF (left column) and cryo-arrested twice with an interval of 5 min without stimulation (middle column) as well as cells chemically fixed without EGF stimulation (red) and 5 min after (blue) stimulation (right column). In the upper two rows, clusters were grouped by the number of molecules they contain. The fraction of molecules per group are shown individually (first row) or as differential plot (second row). In the lower two rows, clusters were grouped by their diameter. The fraction of clusters belonging to each group is shown individually (third row) or as differential plot (fourth row). (**b**) Average node degree (representing the number of neighbors per cluster) histogram for the inter-cluster arrangement. Data represented as mean ± s.d.; n=8 cells for each condition.



Supplementary Figure 8

Comparative cluster analysis of EGFR between the central and peripheral area of the basal membrane.

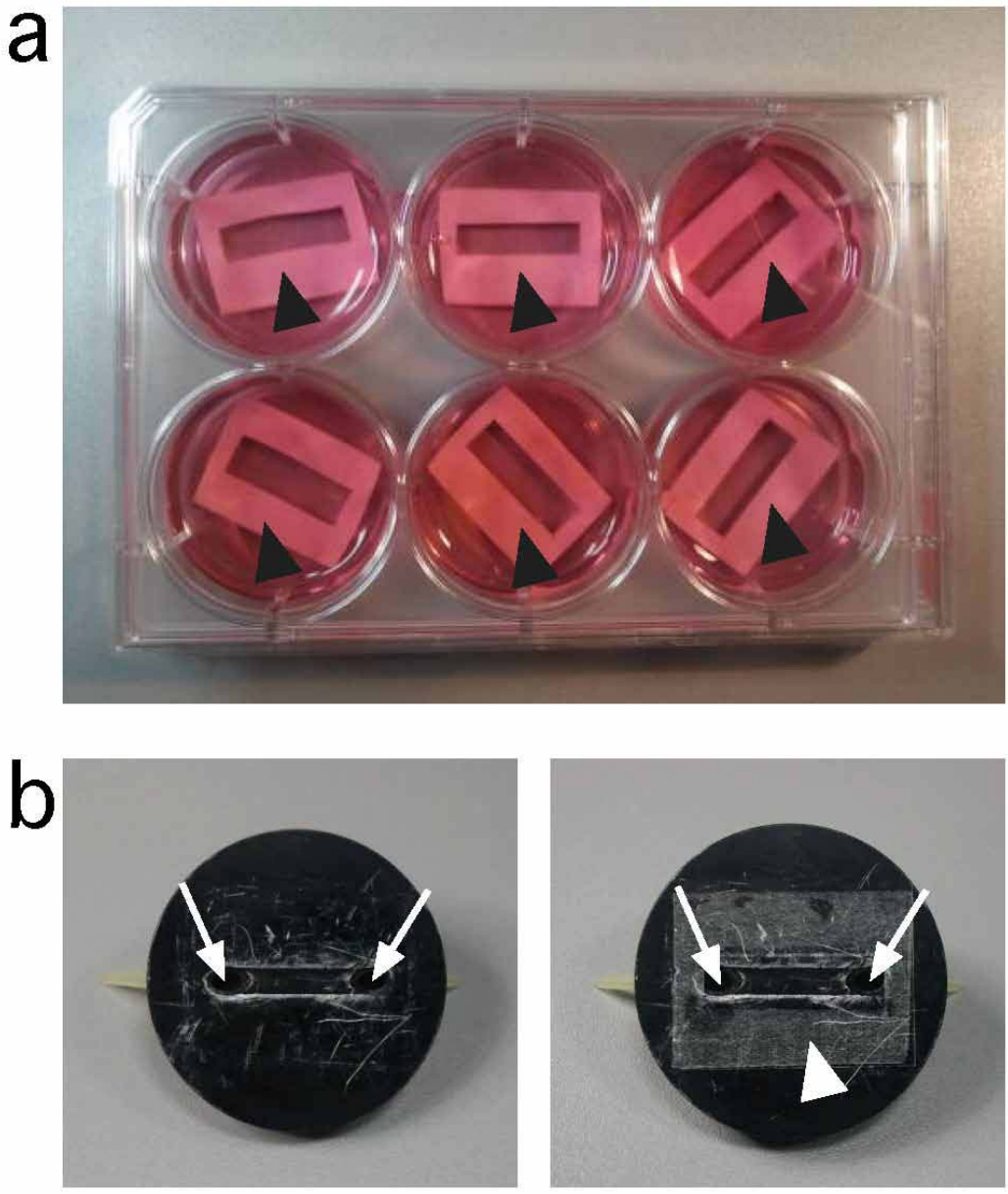
(**a**) Representative example showing the localization of transiently expressed EGFR-mEos2 to the inner (orange) and outer (green) regions of the same cryo-arrested HeLa cell before and 5 minutes after EGF stimulation. Clusters are grouped according to their diameter. The ratio between outer and inner regions of the number of molecules and molecular density per cluster is shown in (**b**) and (**c**), respectively. Data represented as mean ± s.d; n=10 cells.



Supplementary Figure 9

LIFEA2 activity patterns imaged by FLIM.

(**a**) Confocal FLIM measurements of LIFEA2 activation in Cos7 cells upon stimulation with pre-clustered ephrinA1-Fc (2 µg mL^{-1}) for the indicated time (min) at 37°C. Upper row: representative images of LIFEA2 average fluorescence lifetime (τ) at 37°C, lower row: representative images of LIFEA2 average fluorescence lifetime (τ) at -45°C. Scale bars: 20 µm (**b**) Example of a Cos7 cell cryo-arrested 5' after stimulation with ephrinA1-Fc (2 µg ml^{-1}). Left: Spatial binning into 4 concentric radial bins of equivalent area (blue: bin 1 represents the area closest to the plasma membrane; dark red: bin 4 represents the innermost area around the nucleus). Right: fluorescence intensity image of LIFEA2. (**c, left**) Representative average lifetime maps (τ) of LIFEA2 on endosomes in Cos7 cells 5 min and 20 min after stimulation. Endosomes were identified from Fourier transformation of fluorescence intensity images (see Online Methods). (**c, right**) Corresponding α-maps. Scale bars: 20 µm (**d**) Deconvolved confocal FLIM z-scan through a representative HeLa cell cryo-arrested 5 min after stimulation with pre-clustered ephrinA1-Fc. Upper row: LIFEA2 donor (mCitrine) photon count, lower row: corresponding. α-maps of representative slices out of a total of 36. Total acquisition time 37'. Scale bars: 10 µm



Supplementary Figure 10

Mounting of the sample to the stage.

(a) Cover slides sticking to double-sided sticky tape with release liner remaining (black arrow heads) are placed into a 6-well dish and covered with cell culture medium containing cells. The cells are cultured in this arrangement for at least 24 h. **(b)** Anodized aluminum flow-through chamber from the bottom without (left) and with the cover slide (right) glued to it. The channel as well as the medium in- and outlets (white arrows) are not covered by the double-sided sticky tape (release liner removed; white arrow head).