Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information for:

Profiling protein-protein interactions for single cancer cells with in situ lysis and co-immunoprecipitation

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This PDF file includes:

Supplementary Methods

Fabrication of PDMS layer with cell capture array. The mold for the PDMS microfluidic layer with hydrodynamic single cell capture arrays was fabricated by two-step photolithography using SU-8 as a negative photoresist. First, SU-8 2002 (MicroChem) was spin-coated to have a thickness of 2 μm and patterned on a Si wafer to make gaps for cell capture (Fig. S1). Then, SU-8 3050 was spin-coated on the patterned Si wafer to have a thickness of ~22 μm and patterned as cell trap arrays. PDMS mixture (Sylgard 184, Dow Corning, 10:1) was poured on the mold and cured in an oven at 65°C. The PDMS layer was peeled off from the mold and the inlets and outlets were punched with a 1.5-mm-OD biopsy punch (IntegraTM MiltexTM Biopsy Punch). To passivate PDMS surface, PDMS blocks were immersed in 3 % (v/v) Pluronic F-68 (P5556, Sigma) solution overnight at 65°C. After treatment, the blocks were rinsed with DI water and dried for later use.

Glass coverslips for single-molecule imaging. Detailed procedures for preparing PEG-coated glass coverslips were described in the previous study[17]. Briefly, bare glass coverslips (#48393-081, VWR) were rinsed with DI water and cleaned in piranha solution (60 ml of concentrated sulfuric acid mixed with 30 ml of hydrogen peroxide) in a glass staining jar for 1 h. After cleaning, coverslips were then reacted with aminosilane solution (Amino-silane:Acetic Acid:MeOH = 1:5:100, #A0700 United Chemical Technologies) for the coupling of PEG layer. After rinsing with DI water and drying with a stream of nitrogen gas, the coverslips were incubated with 10% (w/v) PEG solution (biotinylated PEG:unlabeled PEG=5:95) at room temperature for 6 h. After incubation, coverslips were again rinsed by water and dried with a nitrogen stream. The PEG-coated coverslips were stored at −20°C for

later use.

Microfluidic device configuration. A fabricated PDMS layer with cell capture array and a PEG-coated glass coverslip were assembled together. The air pressure in the air conduit for reversible sealing of the device assembly was dropped with a vacuum pump (2546C-10, Welch) connected to the air channel via stainless steel catheter coupler (SC23/8, Instech Laboratories). Polyethylene tubing (T23-181-487, Tygon) filled with distilled water was connected to the sample channel ports via 17-gauge needles. Sample liquids were drawn from a reservoir (a pipette tip inserted into the inlet port) into the channel by a syringe pump (PHD22/2000, Harvard apparatus) connected to the outlet.

Cell culture and single-cell suspension. PC9 cell lines were purchased from ATCC. These lung adenocarcinoma cells were grown in RPMI1640 (22400-105 Life technologies) supplemented with 10% fetal bovine serum (26140-079 Life technologies), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (15140-122 Life technologies). All cell lines were cultured in a humidified incubator in 100-mm culture dish at 37°C and 5% CO_2. To prepare a single-cell suspension, cultured cells in dishes were rinsed with phosphate buffered saline (PBS) and detached from the surface by treating them with trypsin (T#12605010, ThermoFisher) for 7 min. The detached cells were centrifuged to a pellet and re-suspended in 300 μl of freshly prepared cold incubation buffer (3 mM colchicine (# C9754-500MG, Sigma-Aldrich), 10 μg ml^{-1} latrunculin B (# L22290, Life Technologies), and 30 units ml^{-1} DNAse I (M0303S, NEB) in PBS, due to latrunculin B the buffer contains 4% DMSO).

Cell capture and lysis in microfluidic channel. For surface attachment of cells and

extraction of membrane proteins, 0.1 mg ml^{-1} NeutrAvidin (A2666 Life Technologies) and 2 μg ml^{-1} biotinylated EGFR antibody (MS-378-B0 Thermo Scientific) were sequentially passed through the sample channel with a flow rate as slow as 0.8 μl min^{-1} over 10 min (total of 8 μl) with 2 times of PBS washing after each step. For cell capture and lysis, the assembled device was then placed on a cold stage. A freshly prepared suspension of cells was filtered through a cell strainer (70-μm mesh; #352350, BD Falcon) and loaded into the microfluidic channel. Cell trapping was monitored on a bright-field microscope and stopped when a sufficient number of traps were occupied by single cells. After cell capture, the inlet of the channel was washed with 8 μl of incubation buffer for 2 times using pipette then 8 μl of incubation buffer was flowed twice into the channel. After 3hrs, the channel was incubated with unlabeled EGFR antibody (MS-378-P1, Thermo Scientific) for another 30 min to block EGFRs on cell membranes that are not bound to the surface immobilized antibody. Then 8 μl of lysis buffer (50 mM Tris-HCl (pH 7.4) with 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 1% (v/v) Triton X-100) was flown in at 2 μl min^{-1} for 4 times. Additionally, 8 μl of washing buffer (PBS with 2 M NaCl and 1% Triton X-100) was flown through twice at the same flow rate.

Construction and transfection of eGFP-tagged prey proteins. Rat PLCγ SH2 domain cDNA (P10686; amino acids 542–765) was directly isolated with *Bgl*II and *Eco*RI from a Rat cDNA library. The cDNAs for full-length Grb2 (Addgene 46442; P62993) and p85α (Addgene 1399; P26450) were single cut by *Eco*RI (Grb2) and *Bsp*EI (p85α) from their original plasmids, respectively. The eGFP-tagged prey proteins were generated by cloning the cDNAs into pEGFP-C1 (Clontech Laboratories)[20]. Then the plasmid was transfected into HEK293-T cells via electroporation (Neon transfection system, MPK5000 Life technologies).

Typically, 30 μg of plasmid DNA was mixed with 100 μl of HEK293-T cell suspension that contained ~2×10^6 cells. Two 950-V pulses (35-ms duration) were applied to the mixture for electroporation. Transfected cells were cultured again for 24 h, harvested, and centrifuged. Cell pellets were stored at –80°C for later use.

Preparation of eGFP-tagged downstream protein extract. Collected cells were suspended in lysis buffer (50 mM Tris-HCl (pH 7.4) with 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 1% (v/v) Triton X-100) supplemented with 1% (v/v) protease inhibitor cocktail (P8340 Sigma) and phosphatase inhibitor cocktail (P5726 Sigma). This cell suspension was incubated at 4°C for 30 min. Samples were mixed using a pipette every 10 min. Then cell suspension was centrifuged at 15,000×g for 10 min at 4°C. The supernatant containing cytosolic proteins was filtered with a polycarbonate filter (0.2-μm pore) for single-molecule experiments. Concentrations of the expressed downstream protein was measured by fluorometer (PerkinElmer, Enspire 2300) using eGFP fluorescence as described in the previous study[17].

Single-molecule co-IP and immunolabeling. For single-molecule co-IP using the pulled down membrane proteins, the first kind of downstream protein extract ($PLC\gamma_{SH2}$ here) diluted to 30 nM (with regard to eGFP) was loaded into the device. The eGFP-tagged protein solutions also contained 0.03% Triton X-100 to reduce nonspecific adsorption of proteins on the surface. The cell traps were located programmatically, and the EGFR–$PLC\gamma_{SH2}$ PPI on each site was imaged by TIRF in a sequential manner. When the imaging was finished, the channel was washed with 8 μl of washing buffer at 0.8 μl min^{-1} twice, and the second prey protein (Grb2 here) was introduced, and so on. For immunolabeling experiments following

co-IP, another EGFR antibody for immunoblotting (4267 Cell signaling) and Cy3-labeled secondary antibody (111-165-046 Jackson ImmunoResearch) was sequentially introduced, with 5-min incubation for each. Each antibody after the reaction was washed with washing buffer in the same manner co-IP experiments. The resulting fluorescent spots (Cy3) were imaged by TIRF.

Imaging setup. An objective-based TIRF setup for single-molecule fluorescence imaging was built with an inverted microscope (Olympus IX71). For TIR excitation of eGFP and Cy3, blue (473 nm; Spectra-Physics, cat. no. Excelsior 473, 50 mW) and green (532 nm; Coherent, cat. no. COMPASS 215M-50) lasers were used respectively. Single-molecule fluorescence was detected by an electron multiplying charge-coupled device (EM-CCD; iXon Du897D-CS0-#BV Andor technology). Custom LabView software (National Instruments) was used to control a motorized stage with piezo-driven Z-axis (PZ-2150FT ASI) and automate the acquisition process.

Extracting EGFR PPI and EGFR level from fluorescence images. The fluorescence images recorded with an EM-CCD were analyzed by custom software written in Matlab (MathWorks). To monitor the changes in fluorescence from dynamic protein-protein interaction, we routinely recorded images at 20 Hz with 50-ms exposure time. This interval was sufficiently short compared to the typical dwell time for a bound protein: for example, EGFR−PLCγ_{SH2} complex, one of the weakest interactions we measured, showed a mean dissociation time of ~1 s when analyzed in a similar manner to the published methods (refs. 16−17 in the main text). We then averaged five such images (total of 0.25-s interval) to reduce noise and reject spurious events (Fig. S9). Image masks for the trapped cells obtained

from bright-field images (Fig. S10) were applied to extract the fluorescence specifically from the trapped cells. Before extracting signals, the background fluorescence inside the cell mask was subtracted, assuming a parabolic profile for the local background.

Supplementary Figures

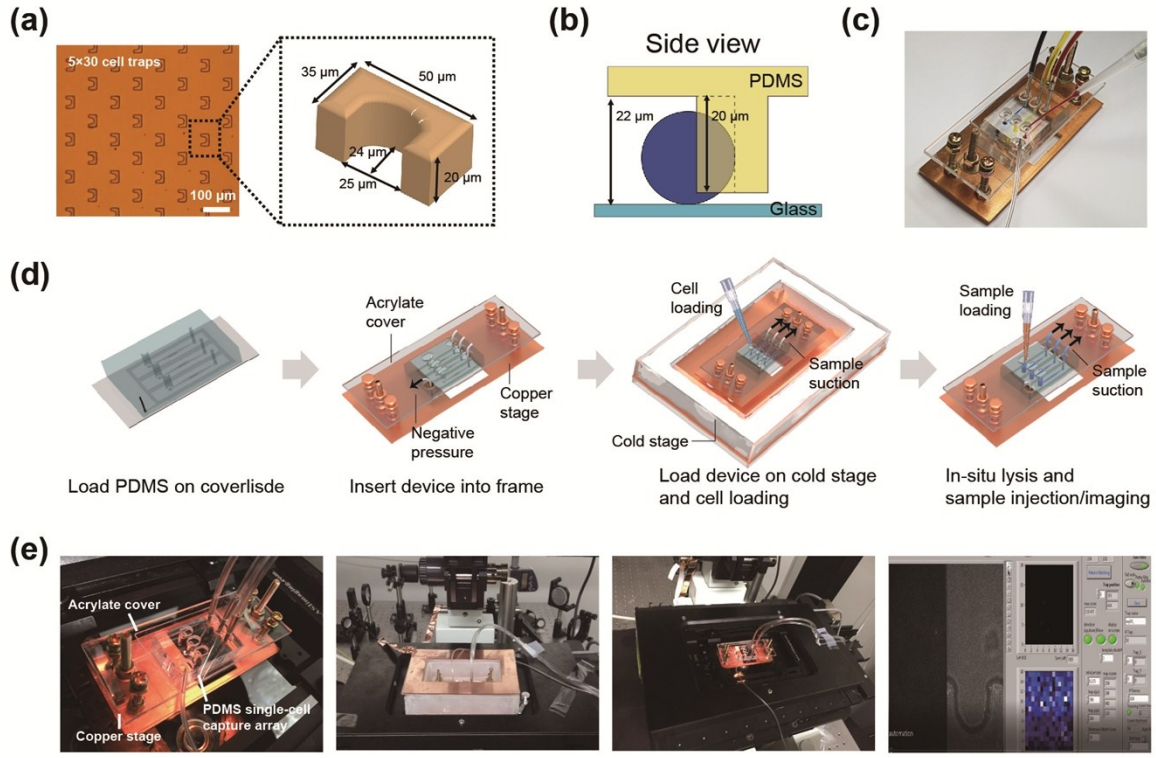
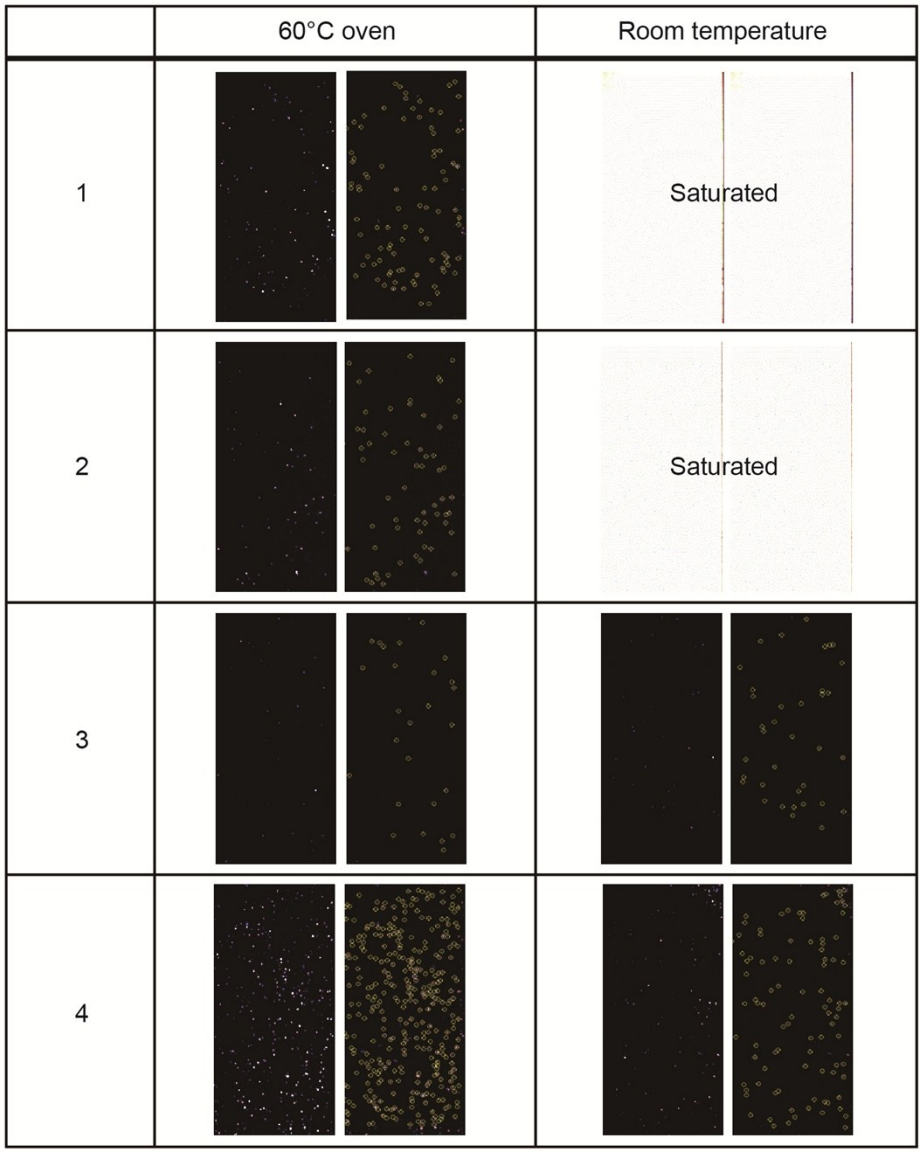


Fig. S1. Design and operation of microfluidic single cell capture device. (a) PDMS microfluidic layer with 5×30 cell traps along the channel and the single cell trap design. (b) Side view of a single cell trap. (c) Picture of the assembled single cell capture array device (d) Experimental procedure. The PDMS layer and the functionalized coverslip are assembled in a frame. The vacuum suction to the air conduit hold the two layers to form microfluidic channels, while the frame allows easy handling of the device and prevent dislocation of the microfluidic channel by external mechanical force. Cells are loaded and captured inside the channel on a cold stage (< 4°C). After on-chip cell lysis, eGFP-labeled downstream prey proteins are injected in series for imaging. (e) Photographs of device setups on a TIRF microscope.

(a)


60°C oven
Room temperature
1
2
3
4
Saturated
Saturated

(b)

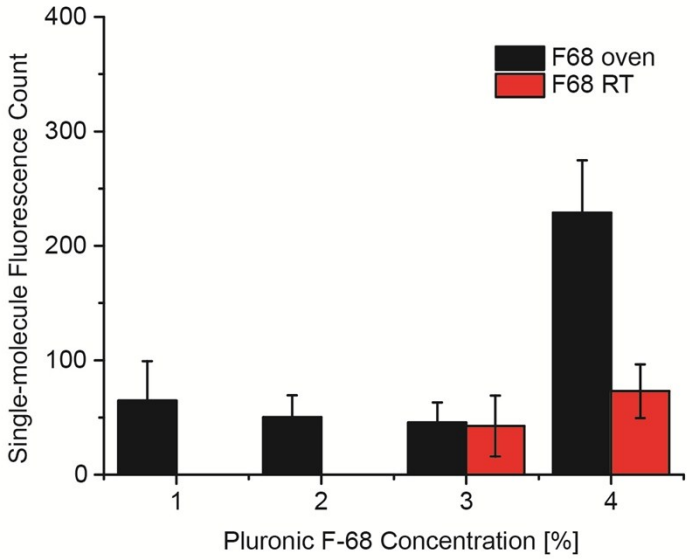
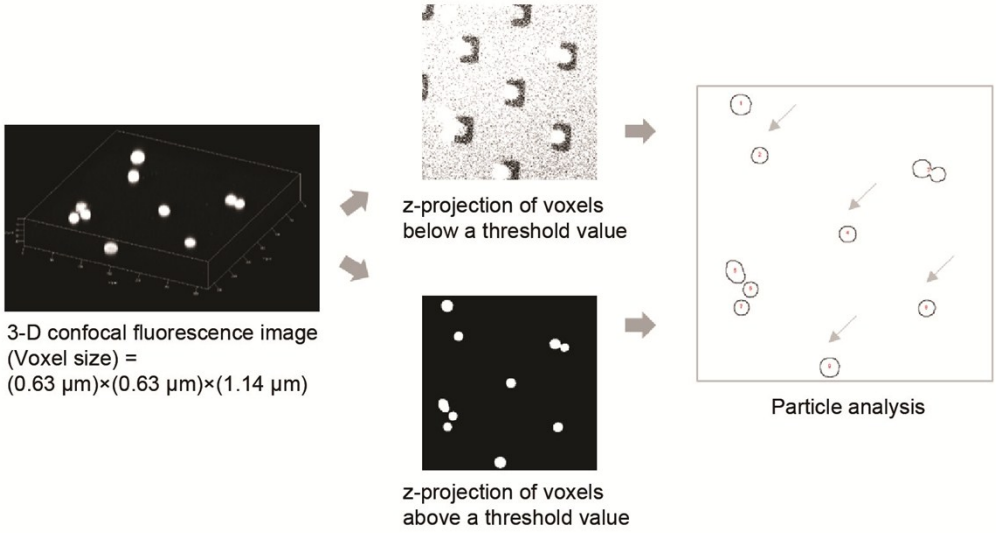

F68 oven
F68 RT
Single-molecule Fluorescence Count
400
300
200
100
0
1
2
3
4
Pluronic F-68 Concentration [%]

Fig. S2. Surface passivation of PDMS. PDMS slabs were immersed in the indicated solutions for 30 min and then washed with DI water. The PDMS slabs were then incubated with Alexa 647 labeled IgG in PBS for 30 min to test nonspecific adsorption of proteins. After rinsing with PBS, the slabs were imaged on a TIRF microscope. (a) Fluorescent images of nonspecifically bound IgG for the indicated conditions. Left images are raw fluorescence images and right images are images with the single-molecule detection (yellow circles). (b) Effects of Pluronic F-68 concentration and incubation temperature on nonspecific binding.

(a)



(b)

	Area [μm²]	Diameter [μm]	# cell
Control bead	87.2 ± 10.2	10.5 ± 3.6	137
Free PC9	285.7 ± 98.1	19.1 ± 11.2	107
Captured PC9	292.3 ± 70.4	19.3 ± 9.5	62

Fig. S3. Cell size distribution. Before imaging, cultured PC9 cells were stained with 1 μM CellTracker Green CMFDA dye (C2925, Thermo Fisher, USA) in RPMI media for 30 min under 37°C and 5% CO_2. Then, the cells were detached with trypsin and re-suspended in DPBS for imaging. For free PC9 cells and control beads, samples were injected into a 150-μm-thick channel formed between two glass surfaces. Imaging was performed on a confocal fluorescence microscope (LSM710, Zeiss) with 20× dry objective. (a) Images and analysis of captured cell shapes with 3D confocal fluorescence microscopy. The binary images obtained by thresholding projected images were analyzed to extract the cross-sectional areas of single cells. To identify single PC9 cells in the trapping device, the weak fluorescence from PDMS traps were used as position markers, and only the well-resolved single cells were subjected to further analysis. (b) Result Table corresponding to Fig. 1c.

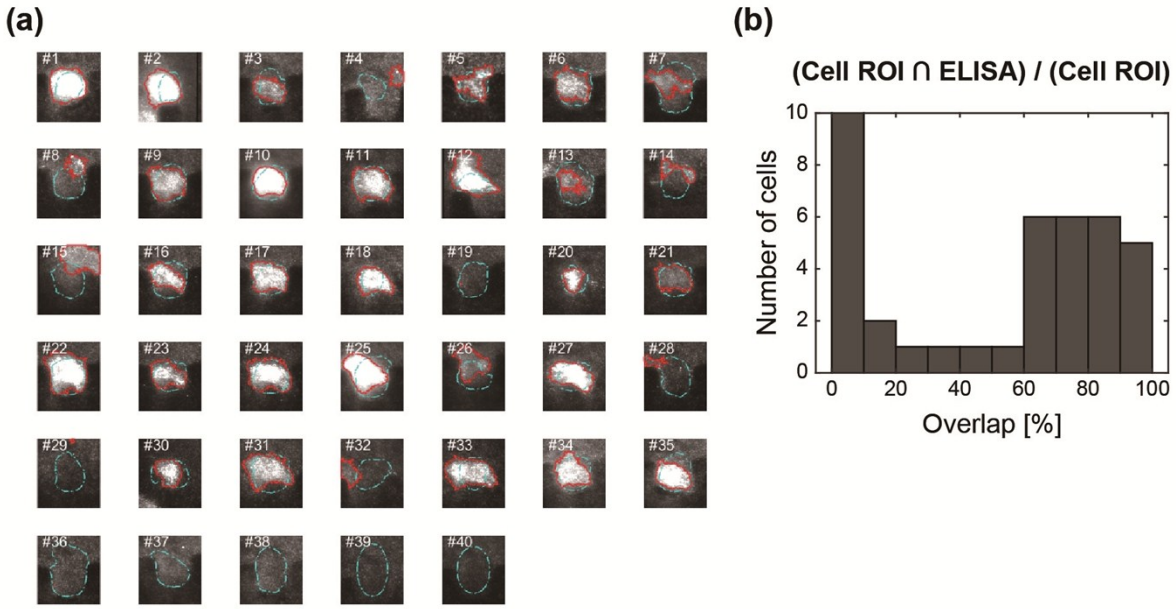


Fig. S4. Snapshot of immunoprecipitated EGFR from control PC9 cells. (a) Clusters obtained by thresholding tEGFR ELISA images (red) and cell ROIs obtained from bright-field images (cyan). #36−#40 were empty traps with pseudo ROI. (b) Histogram of overlap percentage.

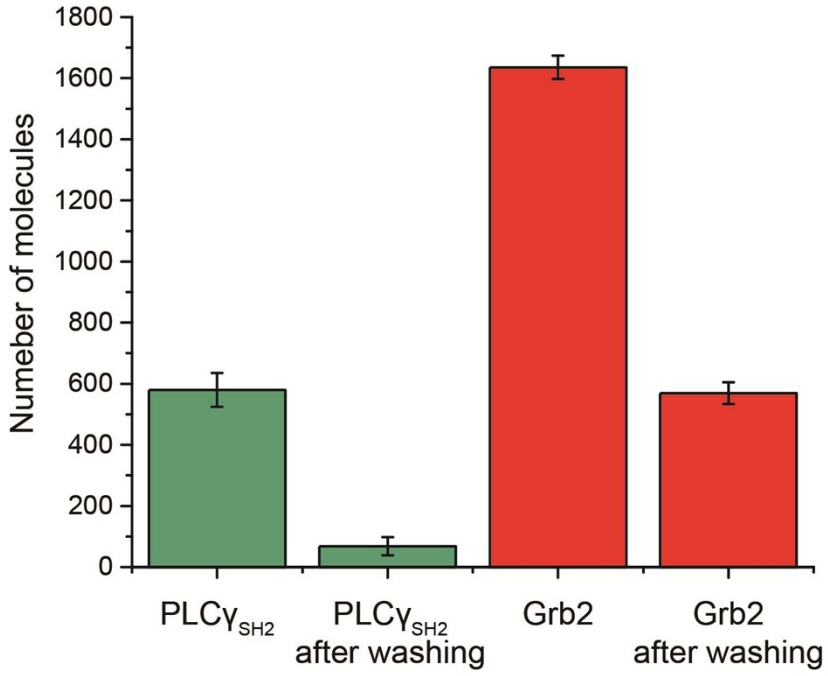


Fig. S5. Residual proteins after a buffer wash. The amount of remnant proteins after washing with buffer was verified by single-molecule counting. On average, 12% of PLCγ_{SH2} and 35% of Grb2 were found to be carried over. Thus, for more accurate quantification of PPI, we subtracted proportionate values from our successive measurements on PLCγ_{SH2}, Grb2, and p85α. However, since the differences in the measured single-cell PPIs were generally substantial (see Fig. 3b in the main text), such correction did not impact the interpretation of the results.

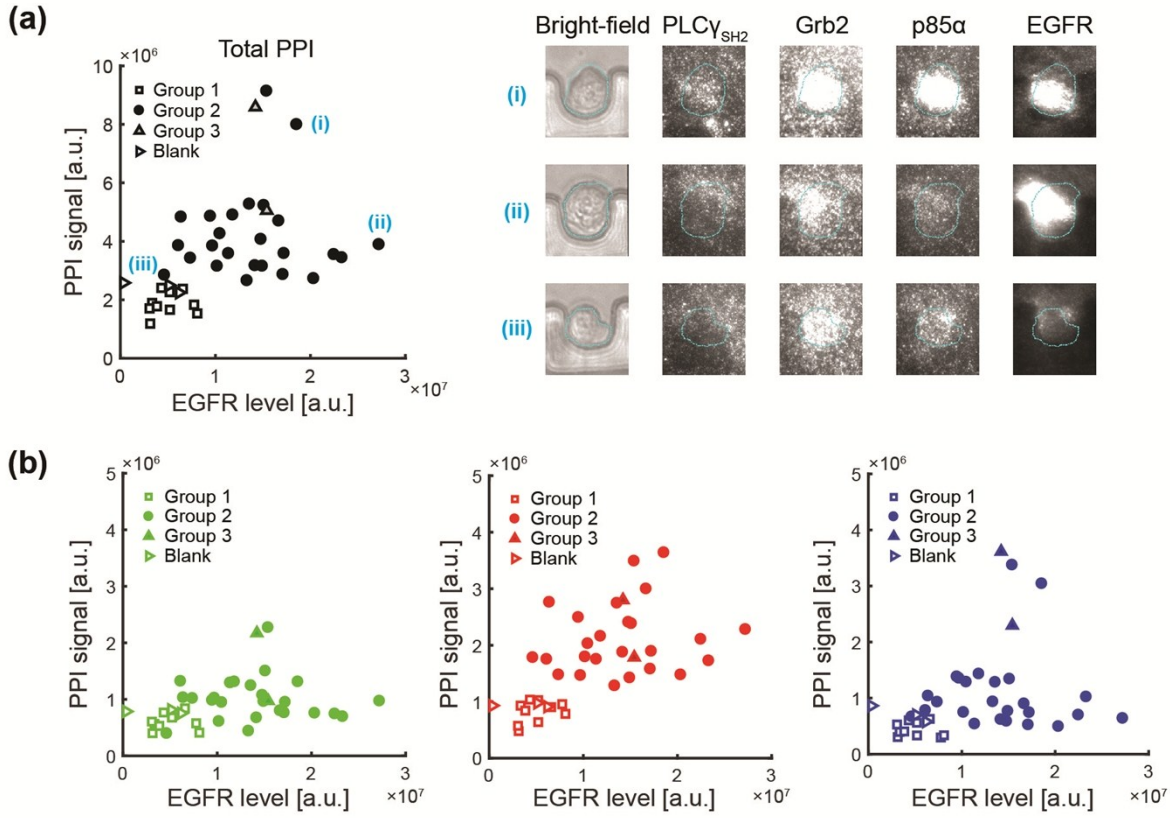


Fig. S6. Dispersion of PPI data. (a) Total PPI vs. EGFR level. Representative images corresponding to the cells (i), (ii), and (iii) are shown on right. (b) PPI signals for individual downstream proteins.

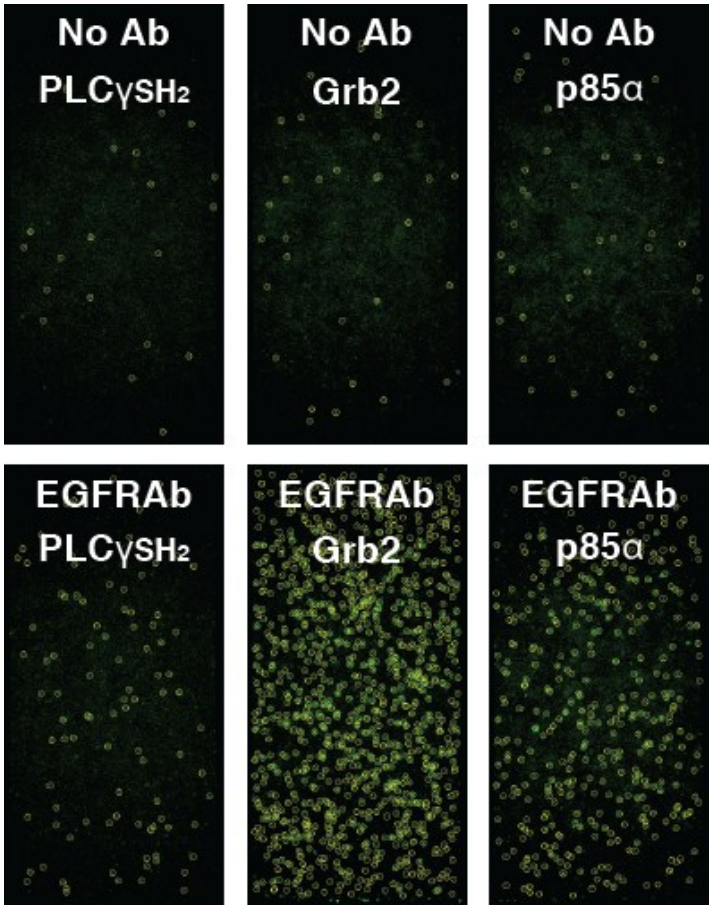


Fig. S7. Comparison of single-cell vs. bulk PPI results. Single-molecule co-IP imaging using a bulk lysate obtained via lysis of ~10^5 PC9 cells. Single molecule co-IP images screen shots corresponding to Fig. 3c.

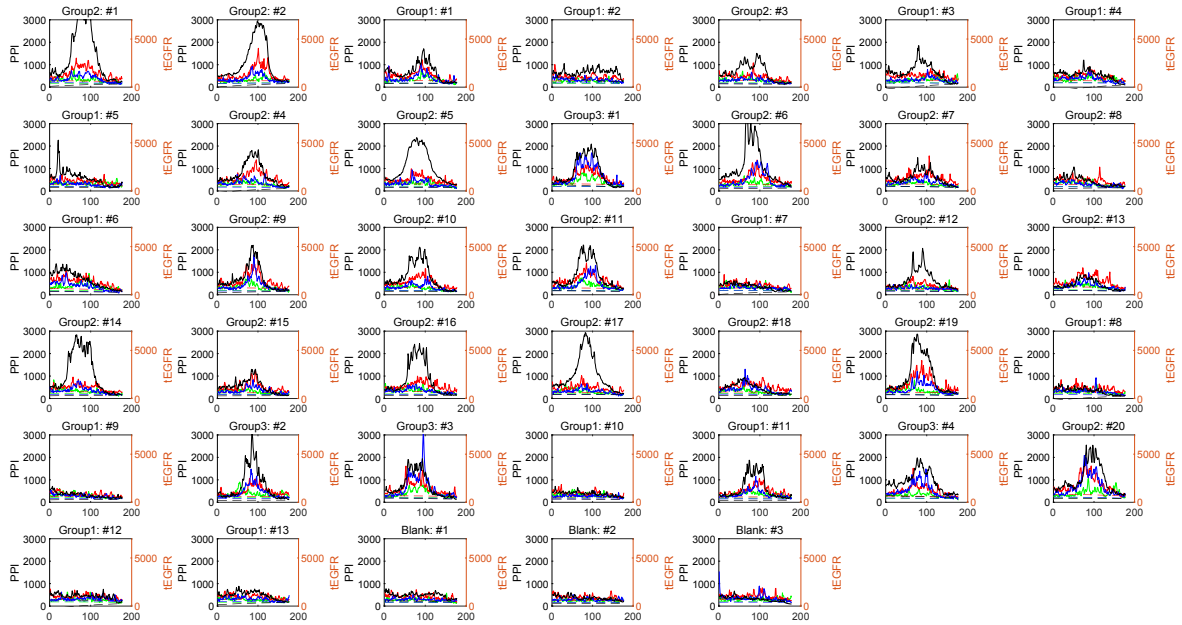
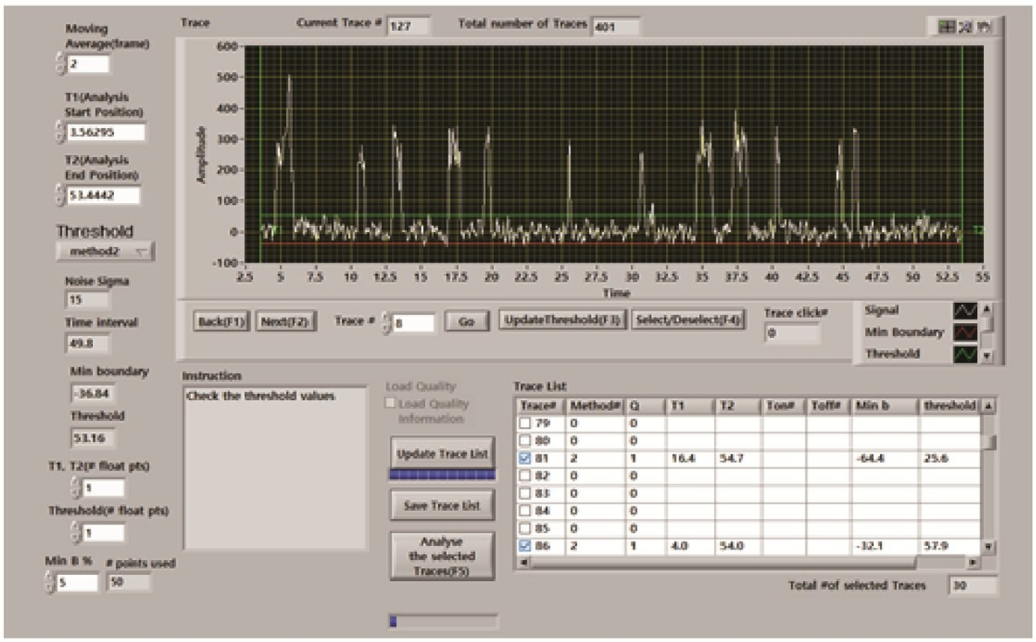


Fig. S8. Cross-section of PPI and ELISA images from PC9 cells. Cross-sections from 37 cells and 3 empty traps are plotted. Black: tEGFR ; Green: PLCγ_{SH2}; Red: Grb2; Blue: p85α.

(a)



(b)

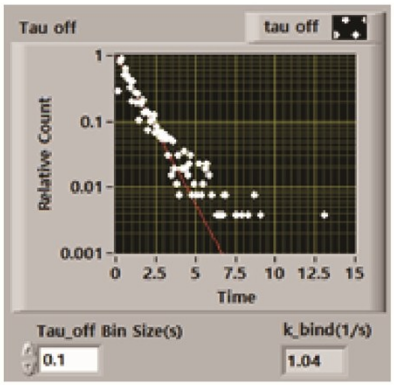


Fig. S9. Real-time trace analysis for EGFR−PLCγ_{SH2} interaction. (a) Snapshot of software for the real-time analysis of EGFR−PLCγ_{SH2} interaction in single-molecule experiments. (b) τ_{off} histogram and calculated k_{bind} (1/s).

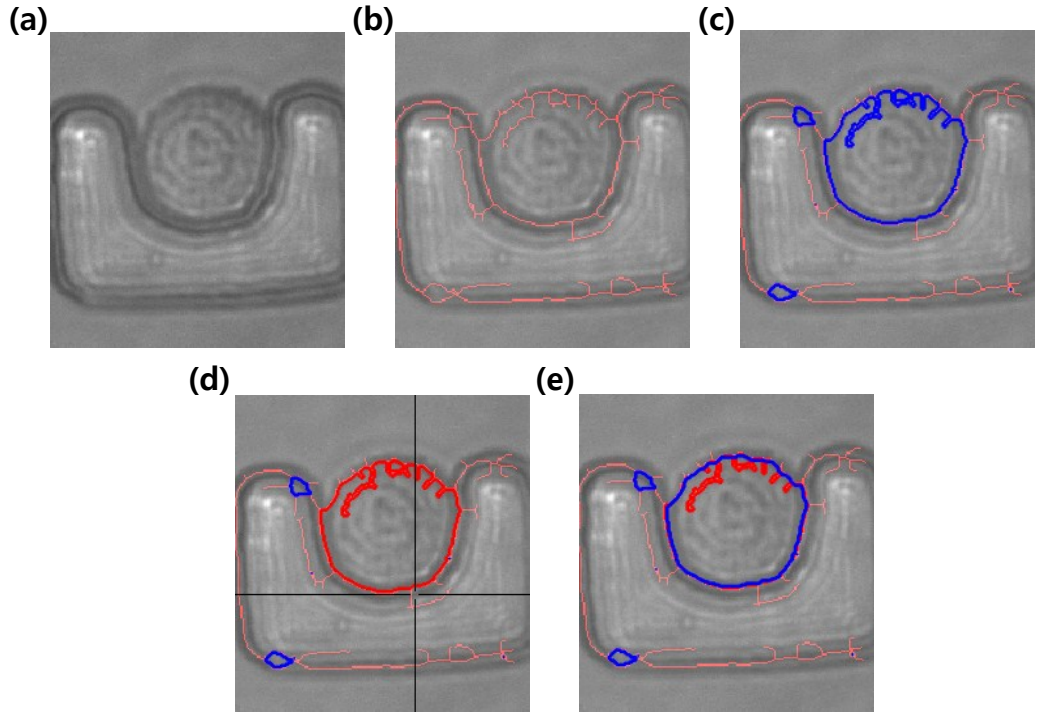
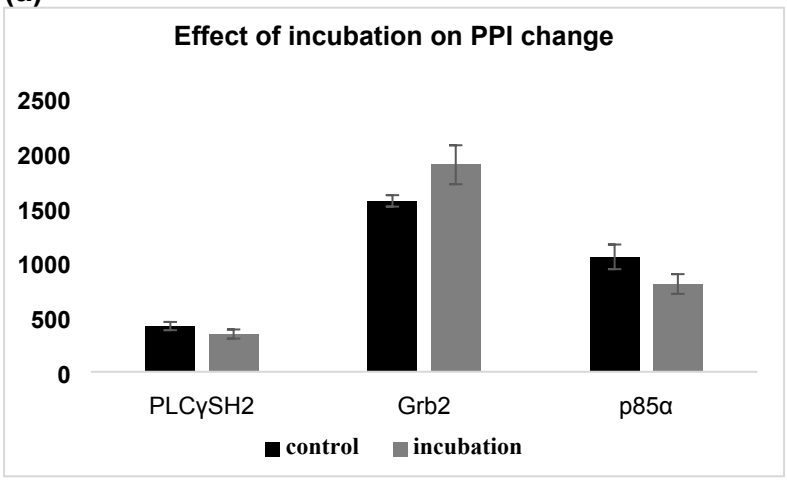


Fig. S10. Generation of image mask (cell ROI) from bright-field image. (a) Original image of a cell trap. (b) Line detection with manual adjustment when necessary (red). In case of empty trap, an arbitrary elliptical boundary is drawn manually. (c) Boundary detection using image segmentation algorithm provided in Matlab (blue). (d) Manual selection of ROIs. (e) Combined ROI generation (blue).

(a)



(b)

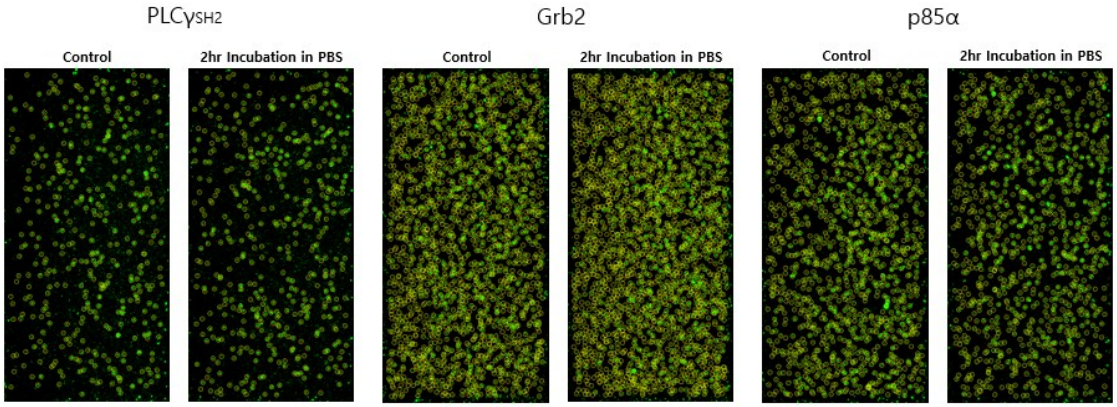


Fig. S11. Effect of washing and buffer incubation on PPI. (a) Differences in PPI change without and with 2-h incubation in buffer after a 10-min washing step. (b) Snapshots of single-molecule data.

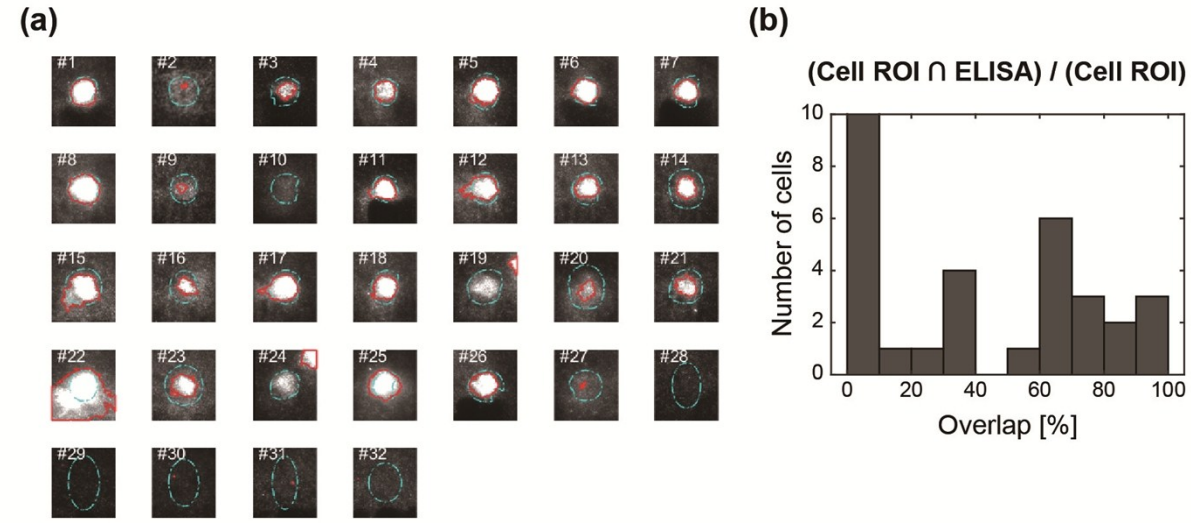


Fig. S12. Snapshot of immunoprecipitated EGFR around cell ROI for drug-treated PC9 cells. (a) Clusters obtained by thresholding tEGFR ELISA images (red) and cell ROIs obtained from bright-field images (cyan). #27−#32 were empty traps with pseudo ROI. (b) Histogram of overlap percentage.

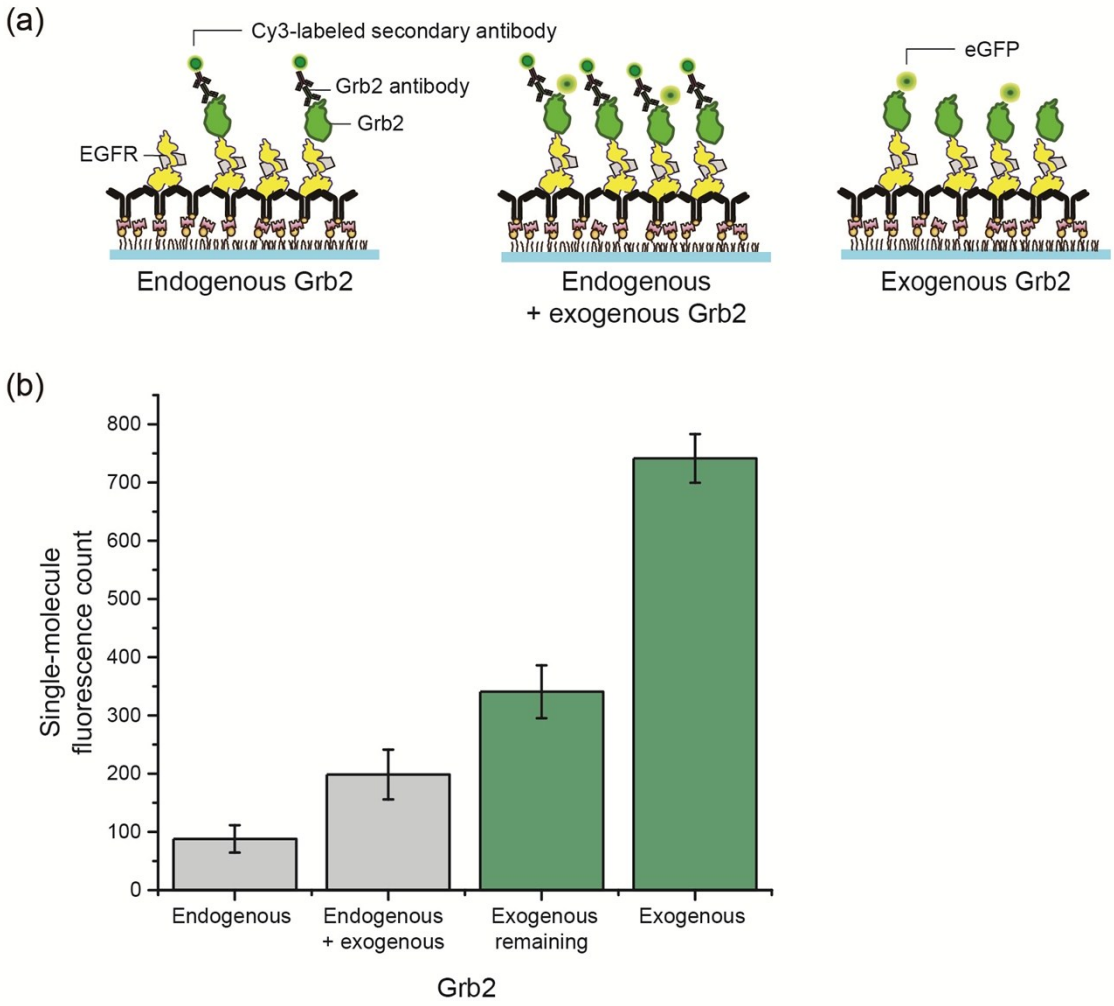


Fig. S13. Estimation of endogenous Grb2 pulled down with EGFR. (a) Schematic of estimating the amount of endogenous Grb2. We performed single-molecule immunolabeling of Grb2 on EGFR pulled downed from PC9 cells. Exogenous eGFP-Grb2 was also added and mildly washed by PBS so that the added Grb2 would remain on the surface with endogenous Grb2. (b) By solving the equations below, we obtained the endogenous Grb2 count of 271, sufficiently smaller than the exogenous eGFP-Grb2 count of 741.

1) $\alpha_{labeling}$×(Endogenous Grb2) = Immunolabeled Grb2 (endogenous)

2) $\alpha_{labeling}$ × (Endogenous Grb2 + Remaining eGFP-Grb2) = Immunolabeled Grb2 (endogenous + exogenous) ($\alpha_{labeling}$: immunolabeling efficiency)