Supporting Information

Oligomerization-Enhanced Receptor-Ligand Binding Revealed by Dual-Color Simultaneous Tracking on Living Cell Membranes

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Experimental

Plasmid Construction.

The DNA fragments encoding full-length CCR5-mNeonGreen were subcloned into the *Hin*dIII and *Xba*I sites of pcDNA3.1(+) (Thermo Fisher Scientific, USA), yielding the CCR5-mNeonGreen expression plasmids. The DNA fragments encoding full-length CCL5-SNAP were subcloned into the *Nco*I and *Xho*I sites of pET-28a(+) (Novagen, USA), yielding the CCL5-SNAP recombinant protein expression plasmids. All the plasmids were confirmed by DNA sequencing.

Protein Expression and Purification.

Escherichia coli BL21(DE3) cells containing the CCL5-SNAP recombinant protein expression vector were grown overnight at 37°C in 100 ml of Terrific Broth containing 100 μg/mL kanamycin. After 1:100 dilution in 1000 ml of fresh medium, cells were grown at 37°C to an optical density of OD_{600}=0.6. Induction of expression was performed overnight with 0.5 mM IPTG at 25 °C. Bacteria were pelleted at 8,000 ×*g* for 10 min at 4°C, and then resuspended in PBS containing 0.2 mM PMSF and 1 mM DTT. After being broken on an ultra-high-pressure homogenizer, the crude extract was obtained by centrifugation at 12,000 g for 20 min at 4 °C, recombinant proteins were subsequently purified via affinity chromatography using HisTrap columns connected to an AKTA Purifier liquid chromatography system (GE Healthcare, USA) according to the instructions of the manufacturer. The purified CCL5-SNAP was collected and analyzed using SDS-PAGE.

CCL5 Ligand fluorescent labeling.

The purified CCL5-SNAP was labeled with the SNAP-Surface Alexa Fluor 546 dyes (NEB, USA) according to the manufacturer's instructions. The protein and the SNAP-tag substrate were incubated in the dark for 30 minutes at 37°C. After the labeling reaction, the unreacted substrate was separated from the labeled CCL5-SNAP fusion protein by gel filtration. The labeling ratio of

fluorescent CCL5 was quantified by measuring the absorbance of the protein solution at 280 nm and 557 nm from the absorption spectra, and according to the Beer-Lambert Law, A=εbC. As shown in Figure S8, the absorption spectra of CCL5, AF546, and CCL5-AF546 can be obtained. The extinction coefficient of AF546 at 280 nm can be calculated according to its reported extinction coefficient at 557 nm and the ratio of its absorbance at 557 nm and 280 nm, respectively. Since the absorption of CCL5 at 557 nm is negligible, the concentration of AF546 in the CCL5-AF546 sample can be determined according to its absorbance and extinction coefficient of AF546 at 557 nm. The concentration of CCL5 in the CCL5-AF546 sample can be calculated with the known extinction coefficient of CCL5 and the absorbance of CCL5 at 280 nm, which is the total absorbance of CCL5-AF546 subtracted by the contribution of AF546 at 280 nm. The latter can be estimated according to the concentration of AF546 and the extinction coefficient of AF546 at 280 nm. The labeling efficiency is calculated with equation $E = C_{AF546}/C_{CCL5}$, where C_{AF546} and C_{CCL5} are the concentration of AF546 and CCL5 in the CCL5-AF546 sample.

Cell Culture and Transfection.

CHO-K1 (CHO) cells (Type Culture Collection of Chinese Academy of Sciences, China) were cultured in F-12K Medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C with 5% CO^2. Cells were growing in a 35-mm glass-bottom dish (NEST Biotechnology, China), and were transfected with 0.2 μg/mL CCR5-mNeonGreen plasmids in the serum-reduced and phenol red-free Opti-MEM (Thermo Fisher Scientific, USA) using lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Generation of CCR5 Stably Transfected CHO Cell Lines.

At 24 h after transfection, the cells were transferred to a selection medium containing 700 μg/ml G418 (Thermo Fisher Scientific, USA). CHO cell lines stably transfected with CCR5 were obtained via single-cell cloning by serial dilution in 96-well plates in the presence of G418 selection antibiotic. After 3 weeks of G418 selection, CCR5 expression levels were assessed by dot blot, then the cells were imaged by confocal microscope and TIRFM.

Chemotaxis Assay.

Chemotaxis assay was assessed using a 6.5 mm polycarbonate membranes transwell chamber with 8 μm pores (Corning, USA). 100 μL of the cell suspension (10^6 cells/mL) suspended in DMEM containing 0.5% (v/v) BSA was added to the upper wells, and 50 nM CCL5 (PeproTech, USA) diluted in the same medium was added to the lower wells. The numbers of cells migrating to the lower wells were quantified using crystal violet staining after 5 h normal culture.

Ligand Treatments.

For the ligand stimulation experiments, the transfected cells which were ready for fluorescence imaging were added with 0nM, 100nM, 200nM, and 500nM CCL5 in the minimal medium for 15 min at 4 °C before fluorescence imaging.

Single-Molecule Fluorescence Imaging.

CCR5 stably transfected CHO cells were seeded into a 35-mm glass-bottom dish (NEST Biotechnology, China) before imaging. Single-molecule fluorescence imaging was performed with a TIRFM based on an inverted Nikon Ti series microscope (Nikon, Japan) equipped with a 488nm and a 532nm solid-state laser (Cobolt, Sweden), a total internal reflective fluorescence illuminator, a 100×/1.49 NA Plan apochromat TIR objective (Nikon, Japan), and an electron-multiplying charge-coupled device (EMCCD) camera (DU897, Andor, UK). mNeonGreen was

excited using the 488 nm laser which was reflected to the sample by a Di02-R488 dichroic mirror (Semrock, USA). The collected fluorescent signals of CCR5-mNeonGreen were passed through a 520/35 filter (Semrock, USA) before being directed into the EMCCD camera. The gain of the EMCCD camera was set as 300. Movies of 300-600 frames were acquired for each sample, at a frame rate of 10 Hz. Single-molecule fluorescence images are detected by an Andor Technology iXon + DU-897E electron-multiplying CCD camera, after a 2× further magnification, yielding a pixel size of about 107 nm.

Dual-color TIRFM.

For the dual-color TIRFM, CCR5-mNeonGreen was excited using a 488 nm laser and CCL5-AF546 was excited using a 532nm laser, and those two lasers were reflected by a multi-band dichroic mirror Di03-R405/488/532/635-t1-25x36 (Semrock, USA). Before being captured by the EMCCD camera, the emission signals were further passed through a W-VIEW GEMINI image splitting optics (Hamamatsu, Japan) with filters for spectral separation of mNeonGreen and Alexa Fluor 546. The W-VIEW GEMINI is an image-splitting optics that provides one pair of dual-wavelength images separated by a dichroic mirror onto a single camera. Specifically, a Di03-R532 dichroic mirror was used to separate the mixed emission signals to a short-wavelength path and a long-wavelength path, a 520/35 filter was used in the short-wavelength path, and a 585/29 filter (Semrock, USA) was used in the long-wavelength path.

Image Analysis and Single-particle Tracking.

For analysis of single-molecule fluorescence intensity in a movie acquired from living cells, the background fluorescence was first subtracted from each frame using the rolling ball method in Image J software (National Institutes of Health, USA). Then the fluorescent spots of 5 × 5 pixels size (530nm × 530nm) in each frame of the movie were detected and tracked using an Image J

plugin called TrackMate which is a software tool for automated, and semi-automated particle tracking[1].

Receptor Oligomeric Status Statistic.

Three sets of parallel replicate cells at each expression level were imaged under TIRFM to characterize the CCR5 oligomeric status. As shown in Figure S5, fluorescence intensity trajectories analyzed from individual diffraction-limited spots showing one-step, two-steps, three-steps, and four-steps photobleaching and represent CCR5 monomer, dimer, trimer, tetramer respectively. Three sets of data with 1000, 2000, and 5000 spots, detected from more than ten microscope fields, more than 15cells, were analyzed. T-test shows that the results obtained from these three sets of data have no significant difference, demonstrating that statistical saturation has been reached.

Figures

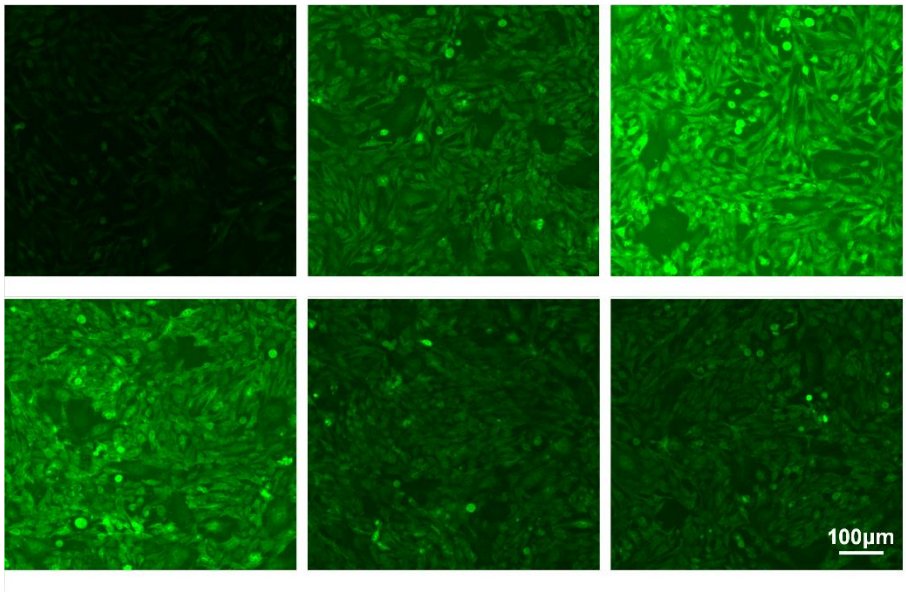

Figure S1. Typical wide-field fluorescence imaging of CCR5 stably transfected CHO cell lines with different CCR5 expression levels.

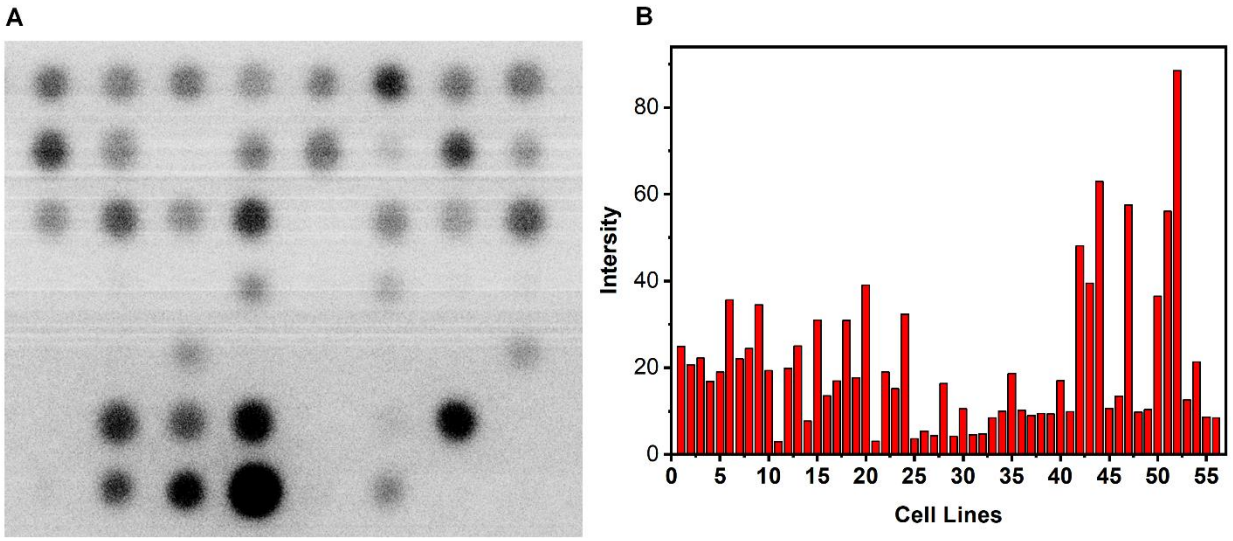

Figure S2. Characterization of the CCR5 expression level of the stably transfected CHO cell lines. (A) CCR5 expression level dot blots results. (B) Grayscale analysis of CCR5 expression level dot blots.

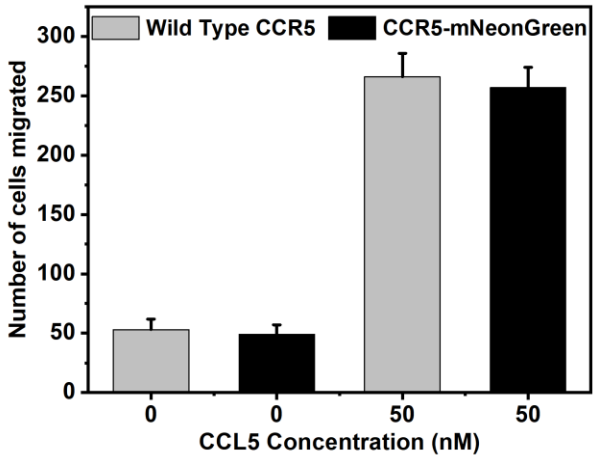

Figure S3. CCR5 transfected CHO cells chemotaxis assay trigged by CCL5.

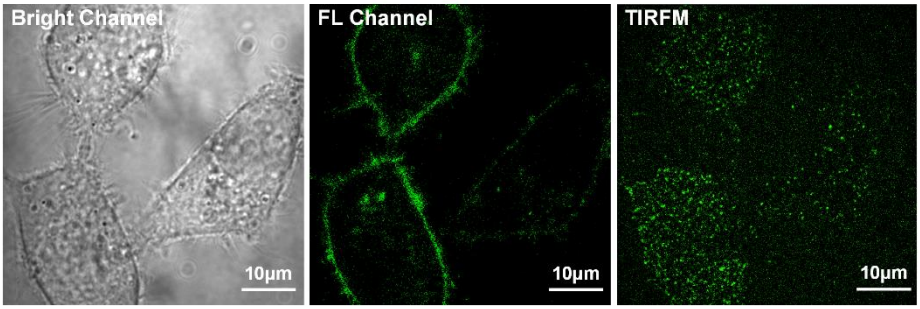

Figure S4. Images of CCR5-mNeonGreen stably transfected CHO cells under confocal microscopy and TIRFM.

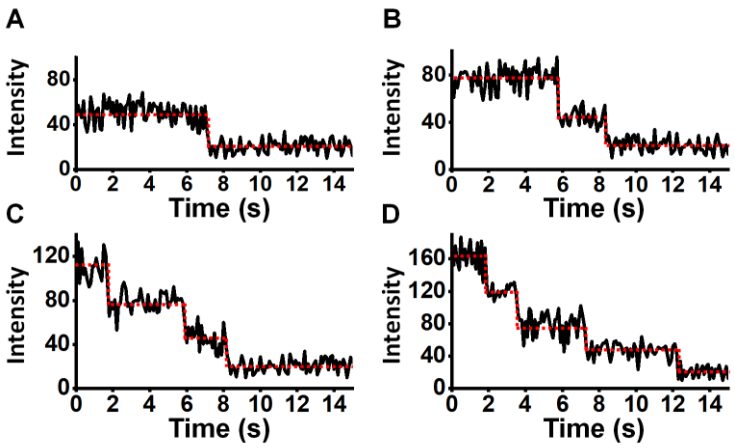

Figure S5. Time courses of mNeonGreen intensity show different steps of photobleaching. (A-D) Representative fluorescence intensity trajectories from individual diffraction-limited spots showing one-step, two-steps, three-steps, and four-steps photobleaching respectively.

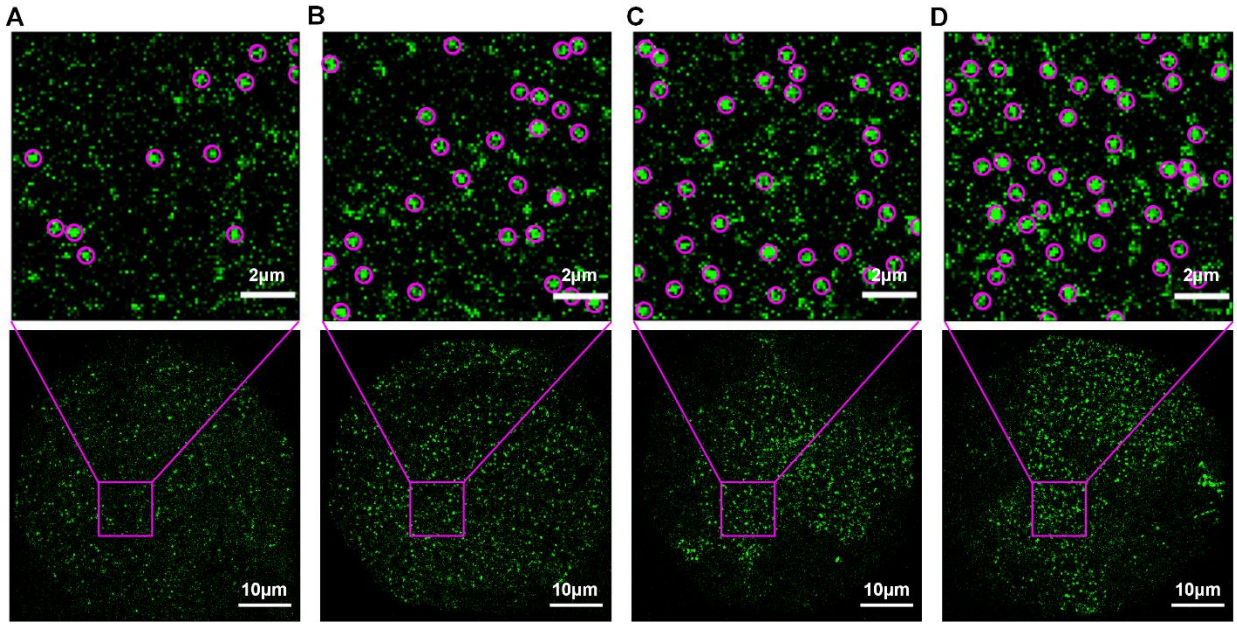

Figure S6. TIRFM imaging of the CCR5 stably transfected CHO cell lines with different CCR5 expression levels. (A-D) CCR5 is expressed on the CHO cell membrane at the density of 0.11, 0.23, 0.34, and 0.44 particles/μm^2 respectively.

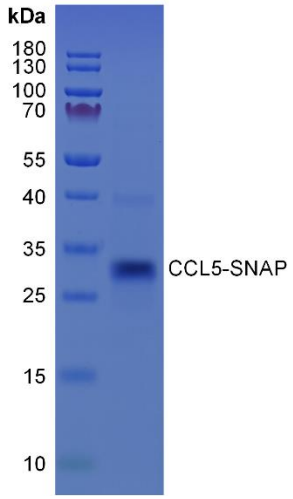

Figure S7. SDS-PAGE analysis of purified CCL5-SNAP protein.

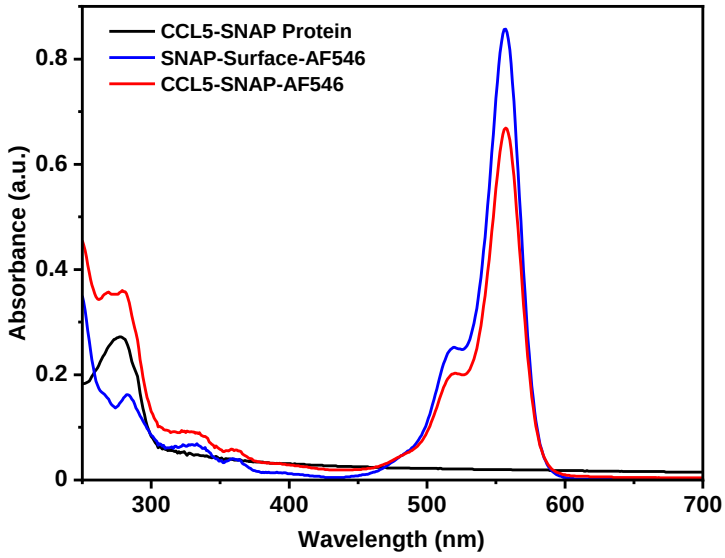

Figure S8. The absorption spectra of fluorescent ligand. The red line is the absorption spectra of the labeled CCL5-AF546 ligand. The black line and the blue line are the absorption spectra of CCL5 ligand and AF546 dye at similar concentrations respectively.

(1) Tinevez, J. Y.; Perry, N.; Schindelin, J.; Hoopes, G. M.; Reynolds, G. D.; Laplantine, E.; Bednarek, S. Y.; Shorte, S. L.; Eliceiri, K. W. TrackMate: An open and extensible platform for single-particle tracking. *Methods* **2017,** *115*, 80-90.