Protein Diffusion

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Analysis of Interactions between the Epidermal Growth Factor Receptor and Soluble Ligands on the Basis of Single-Molecule Diffusivity in the Membrane of Living Cells**

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Abstract: *We present a single-molecule diffusional-mobility-shift assay (smDIMSA) for analyzing the interactions between membrane and water-soluble proteins in the crowded membrane of living cells. We found that ligand–receptor interactions decreased the diffusional mobility of ErbB receptors and β-adrenergic receptors, as determined by single-particle tracking with super-resolution microscopy. The shift in diffusional mobility was sensitive to the size of the water-soluble binders that ranged from a few tens of kilodaltons to several hundred kilodaltons. This technique was used to quantitatively analyze the dissociation constant and the cooperativity of antibody interactions with the epidermal growth factor receptor and its mutants. smDIMSA enables the quantitative investigation of previously undetected ligand–receptor interactions in the intact membrane of living cells on the basis of the diffusivity of single-molecule membrane proteins without ligand labeling.*

On the surface of a plasma membrane, the interactions of membrane proteins with ligands are crucial to the communication between the intra- and extracellular environments.[1] Various types of ligand–receptor interactions occur in the membrane of living cells, yet many of these interactions have not been elucidated.[2] Because the crowded and heterogeneous cellular membrane generates unique biochemical conditions for biomolecular reactions,[3] membrane–protein interactions should be investigated in the intact membrane of living cells if their genuine biochemical properties are to be understood.

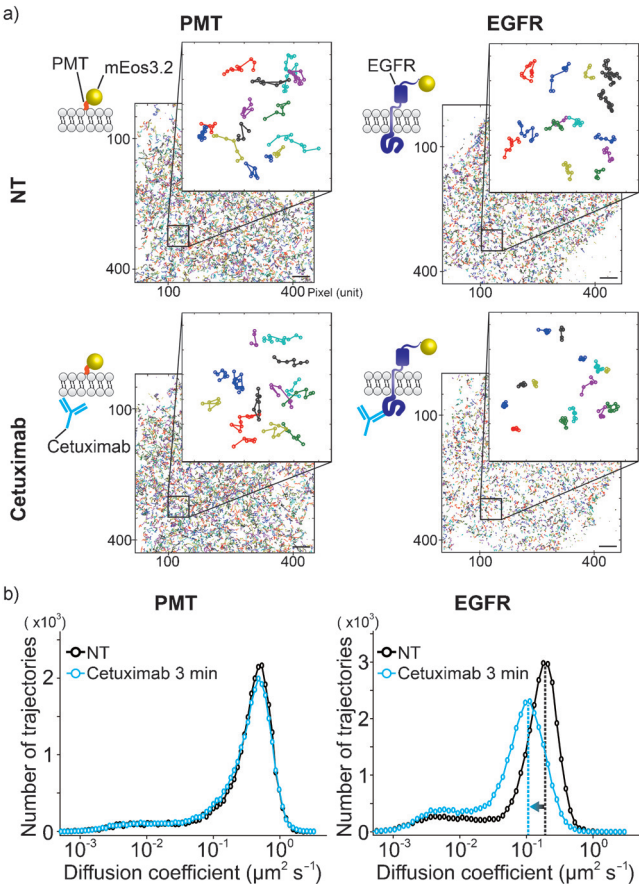

Figure 1. Diffusion-coefficient shift of EGFR as induced by the direct binding of cetuximab. a) Map of accumulated PMT and EGFR trajectories acquired for about 1 min before (NT) and after cetuximab treatment (after 3 min) in a single COS7 cell; 5000 trajectories are shown, and only 14 randomly chosen trajectories are shown in each magnified image. Scale bar: 5 μm. b) Diffusion-coefficient distributions of PMT and EGFR before (black line) and 3 min after (blue line) treatment with cetuximab.

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Diffusion-based imaging techniques have the advantage that the interactions of membrane proteins can be inferred from the intrinsic diffusivity of a membrane protein in living cells.[4] However, it has long been believed that the binding of soluble ligands to a membrane protein contributes negligibly to its diffusivity because the viscosity of a plasma membrane is orders of magnitude higher than that of the aqueous solution surrounding a cell.[5] This hypothesis fundamentally prevents the use of the diffusion-based assays for analyzing ligand–receptor interactions that involve the water-soluble regions of membrane proteins.[6]

Herein, we present a single-molecule diffusional-mobility-shift assay (smDIMSA) for quantitatively analyzing ligand–receptor interactions in the intact membrane of living cells on the basis of the intrinsic diffusivity of membrane proteins. Our assay was developed on the basis of the finding that the single-molecule diffusivity of a membrane protein, as measured by single-particle tracking photoactivated localization microscopy (sptPALM),[7] is significantly shifted by ligand–receptor interactions. smDIMSA probes the interaction between the epidermal growth factor receptor (EGFR) and a water-soluble ligand larger than about 5 kDa without ligand labeling. We quantitatively analyzed the biochemical properties of the interactions of EGFR and its mutants with their antibodies in the membrane of living cells, and we determined the conformation of dimerized EGFR L858R by detecting the positive cooperativity of cetuximab binding to EGFR L858R in a cell membrane.

To obtain single-molecule diffusional dynamics of EGFR, we tagged the C terminus of EGFR with mEos3.2[8] and tracked more than 30000 individual EGFR trajectories (longer than ten frames) by using sptPALM in a single COS7 cell that expresses a low level of EGFR. This number of EGFR trajectories was sufficient to detect a small variation in the diffusion-coefficient distribution: the detection limit for the spatio-temporally averaged diffusion-coefficient change was approximately 5% in a single living cell (see Figure S1 in the Supporting Information). We unexpectedly observed that cetuximab, a monoclonal antibody drug targeting EGFR, significantly decreased the diffusional mobility of the EGFR trajectories immediately (ca. 3 min) after treatment (Figure 1a; see also Movie S1 in the Supporting Information). There was no detectable change in the diffusivity of a plasma-membrane-targeting peptide (PMT) that was designed to localize mEos3.2 on the plasma membrane as a control membrane protein (see Figure S2); thus, this immediate decrease in the EGFR diffusional mobility by cetuximab was specific to EGFR (Figure 1b). The EGFR diffusion-coefficient shift induced by cetuximab was approximately 40% (see Figure S3). No significant batch-to-batch variations in the measurements were observed (see Figure S4). The EGFR diffusion-coefficient shift induced by cetuximab was also observed in cell lines other than COS7, including HEK293, HeLa, and CHO-K1 cells (see Figure S5).

We confirmed that both the signal-to-noise ratio (SNR) for the single-particle detection and the trajectory duration were not altered after cetuximab treatment (see Figure S6). There was also no indication of photodamage to the cell after the measurements (see Figure S7). Furthermore, EGFR aggregation was not induced by cetuximab treatment during our observation time (see Figure S8). We observed that the Fab (fragment antigen-binding) fragment of cetuximab, which binds only one EGFR molecule and does not induce EGFR internalization,[9] specifically produced an approximately 16% diffusion-coefficient shift of EGFR (see Figure S9 and Table S1 in the Supporting Information).

We further confirmed our observation by using different antibodies and membrane proteins. First, we prepared a construct in which EGFR–mEos3.2 was conjugated to a Myc tag at its N terminus (Figure 2a). The anti-Myc antibody and its Fab fragment induced diffusion-coefficient shifts of approximately 42 and 15%, respectively, in a COS7 cell (Figure 2b,c). The binding of the anti-Myc antibody to Myc–EGFR–mEos3.2 had a neutral effect on EGFR signaling (see Figure S10), which implies that the diffusion-coefficient shift was caused directly by the direct binding and not by a secondary effect that occurred after binding. Next, we selected four distinct monoclonal antibodies that were previously reported to target different epitopes of the extracellular domain (ECD) of EGFR: cetuximab,

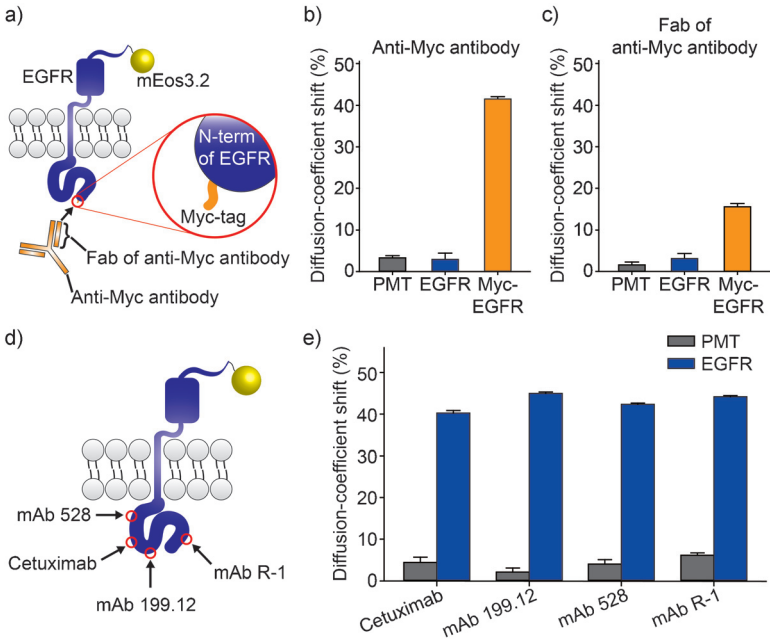

Figure 2. Validation of the diffusional-mobility shift of EGFR as induced by monoclonal antibodies. a) Interactions of an anti-Myc antibody and a Fab fragment of the anti-Myc antibody with myc–EGFR–mEos3.2. b, c) Diffusion-coefficient shifts of PMT, EGFR, and myc-EGFR as induced by the anti-Myc antibody (b) and the Fab fragment (c) of the anti-Myc antibody. d) Schematic representation of the specific binding sites of four anti-EGFR monoclonal antibodies that target the ECD of EGFR. e) Diffusion-coefficient shifts of PMT and EGFR as induced by the four anti-EGFR antibodies. The error bar represents the standard error of the mean (s.e.m.) of a single-molecule population ($n > 10000$).

mAb 199.12, mAb 528, and mAb R-1[10] (Figure 2d). Although the binding of these antibodies to EGFR had different effects on EGF signaling (see Figure S11), these antibodies exhibited similar EGFR diffusion-coefficient shifts immediately after treatment (Figure 2e).

Our finding was also confirmed by using ErbB2 and ErbB3, which belong to the same ErbB receptor family as EGFR but have structural characteristics differ from those of EGFR. We observed that their functionally neutral monoclonal antibodies produced diffusion-coefficient shifts for ErbB2 and ErbB3 of approximately 40 and 39%, respectively (see Figure S12). We further explored the method for the case of highly membrane dependent G-protein-coupled receptors (GPCRs). We examined the β1-adrenergic receptor (β1-AR) and β2-adrenergic receptor (β2-AR) by fusing a FLAG tag to the N termini of these two adrenergic receptors to enable the use of an anti-FLAG monoclonal antibody as a neutral binder. Interestingly, we observed that the anti-FLAG antibody induced approximately 32 and 28% diffusion-coefficient shifts for β1-AR and β2-AR, respectively (see Figure S13).

Next, we investigated the dependency of the amplitude of the diffusion-coefficient shift on the size of the soluble binding proteins. We used an EGFR aptamer (ca. 17 kDa), the Fab and $F(ab')_2$ fragments of cetuximab (ca. 51 and 110 kDa, respectively), and cetuximab (ca. 150 kDa; Figure 3a). The EGFR aptamer shared the binding site of cetuximab (see Figure S14). We determined that the shifts in the EGFR diffusion-coefficient distributions increased as the size of these binders increased (Figure 3b,c). The size sensitivity was 0.26% per kilodalton for the diffusion-coefficient shift, and the binding of water-soluble proteins with a size of up to 5 kDa to EGFR could be resolved; this resolution is beyond that of conventional polyacrylamide gel electrophoresis.[11] We also detected the formation step of the EGFR complex up to approximately 600 kDa (see Figure S15). The biophysical relationship between the diffusion coefficient and the size of the ECD upon the binding of soluble ligands should be further analyzed considering the characteristics of the membrane of living cells[12] (see Figure S16).

One of the important advantages of smDIMSA is that only the specific interactions of a mEos3.2-tagged target receptor with its ligands are measured, regardless of the presence of other proteins or the occurrence of reactions that are irrelevant to the target receptor on a cell membrane. This property is crucial for in vivo assays because various membrane proteins are coexpressed in crowded cell membranes, thus making it difficult to ensure the molecular specificity of the interactions. We evaluated smDIMSA for molecular specificity by using cetuximab and trastuzumab (a monoclonal antibody targeting ErbB2) on the membrane of COS7 cells (Figure 4a). We found that the diffusion-coefficient distribution of EGFR was shifted by cetuximab but not by trastuzumab or an anti-actin monoclonal antibody used as a control (Figure 4a). However, the diffusion-coefficient distribution of ErbB2 was shifted by about 43% by trastuzumab but not cetuximab. These results indicate that the interaction specificities or cross-reactivities of cetuximab and

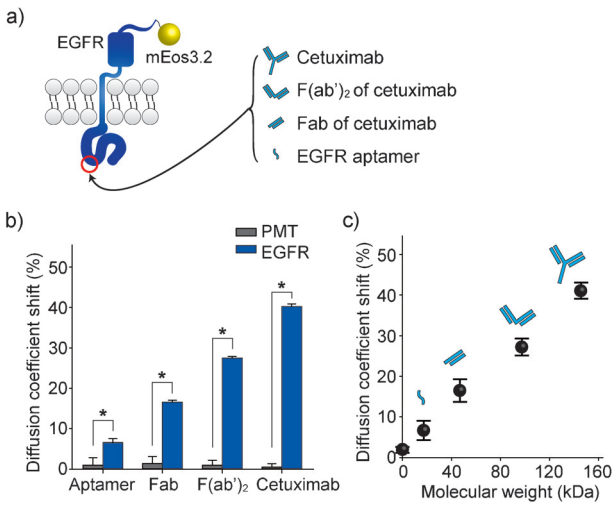

Figure 3. Size-sensitive measurement of EGFR interactions with soluble binders. a) Schematic representation of the size-dependent interaction of EGFR. b) Diffusion-coefficient shifts of PMT and EGFR as induced by interacting partners of various sizes. The error bar represents the s.e.m. of a single-molecule population ($n > 20000$; $*P < 0.001$). c) Relationship between the diffusion-coefficient shift of EGFR and the size of the EGFR interaction partners. The error bar represents the s.e.m. of a single-cell population ($n = 5$).

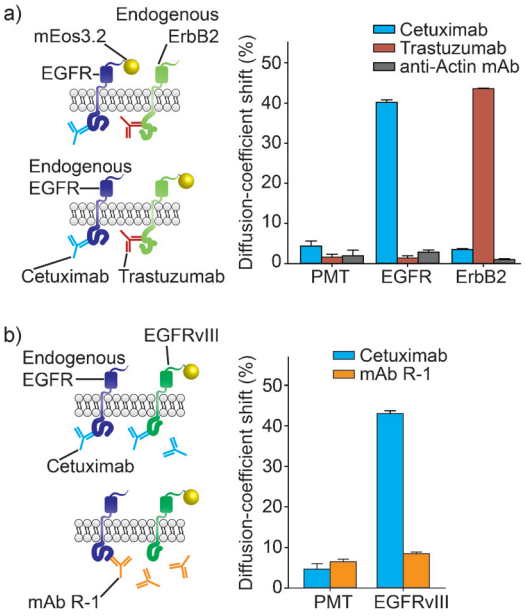

Figure 4. Molecule-specific measurement of ligand–receptor interactions on a cell membrane. a) Left: Schematic representation of the molecule-specific interactions of mEos3.2-tagged EGFR and ErbB2 with their specific antibodies on a cell membrane expressing endogenous EGFR and ErbB2. Right: Diffusion-coefficient shifts of PMT, EGFR, and ErbB2 as induced by cetuximab and trastuzumab. b) Left: Schematic representation of the measurement of the molecule-specific interactions of cetuximab and mAb R-1 with EGFRvIII in the presence of endogenous EGFR expression on the COS7 cell membrane. Right: Diffusion-coefficient shifts of PMT and EGFRvIII as induced by cetuximab and mAb R-1 (right panel). The error bar represents the s.e.m. of a single-molecule population ($n > 10000$).

trastuzumab with EGFR and ErbB2 can be directly measured on the membrane of living cells. To further demonstrate the applicability of smDIMSA for measuring mutant-protein-specific ligand interactions, we exploited the specificity of cetuximab and mAb R-1 for the tumor-associated EGFR variant III mutant (EGFRvIII), in which the N-terminal 267 amino acids of wild-type EGFR (EGFR WT) are deleted. Because the peptide sequence used to generate mAb R-1 corresponds to the deleted region in EGFRvIII, mAb R-1 can bind to EGFR but not EGFRvIII. By using smDIMSA, we observed that mAb R-1 did not bind to EGFRvIII in a COS7 cell membrane (Figure 4b), but did bind to EGFR WT (Figure 2e).

To demonstrate the quantitative aspect of smDIMSA (see the Supporting Information for details), we measured the dissociation constant (K_d) of the interaction between EGFR and cetuximab in a COS7 cell membrane. From the stepwise EGFR diffusion-coefficient shifts that occurred in response to the dose-dependent sequential addition of cetuximab, we extracted the ratios of the cetuximab-bound EGFR at each cetuximab concentration to calculate the K_d value of the interaction between cetuximab and EGFR as well as the binding cooperativity (see Figure S17). The K_d value of cetuximab binding to EGFR was (0.62 ± 0.19) nM, which is similar to the in vitro K_d value of cetuximab binding to the ECD of EGFR[13] (Figure 5a). A Scatchard plot revealed that there was no cooperativity in the binding of cetuximab to EGFR, which is consistent with the results of previous in vitro studies.[13] The cetuximab-bound "EGFR fraction" obtained by smDIMSA can be complementary to the EGFR-bound "cetuximab fraction" measured by labeling cetuximab at the single-cell level.[14]

In contrast to EGFR WT, we found that the interaction between EGFR L858R, which is a mutant frequently found in cancers, and cetuximab on the membrane of COS7 cells exhibited strong positive cooperativity (Figure 5b). Because EGFR L858R was previously reported to form a dimer,[15] we suspected that the binding of cetuximab induced a conformational change in the dimeric EGFR L858R. However, it was unclear how the binding of cetuximab to the ECD of EGFR L858R differed from the binding to the ECD of EGFR WT (Figure 5a) because EGFR L858R contains only a single amino acid substitution in its cytosolic domain and an intact ECD identical to that of EGFR WT. We therefore examined whether this positive cooperativity occurs with mAb 199.12, which binds to an epitope of EGFR L858R that differs from the cetuximab-binding epitope. Surprisingly, no cooperativity of the mAb 199.12 binding to EGFR L858R was observed (Figure 5c), thus indicating that the cetuximab-binding epitope is crucial for the conformational change of dimeric EGFR L858R. Because the extended conformation of EGFR sterically hinders its interaction with cetuximab but not

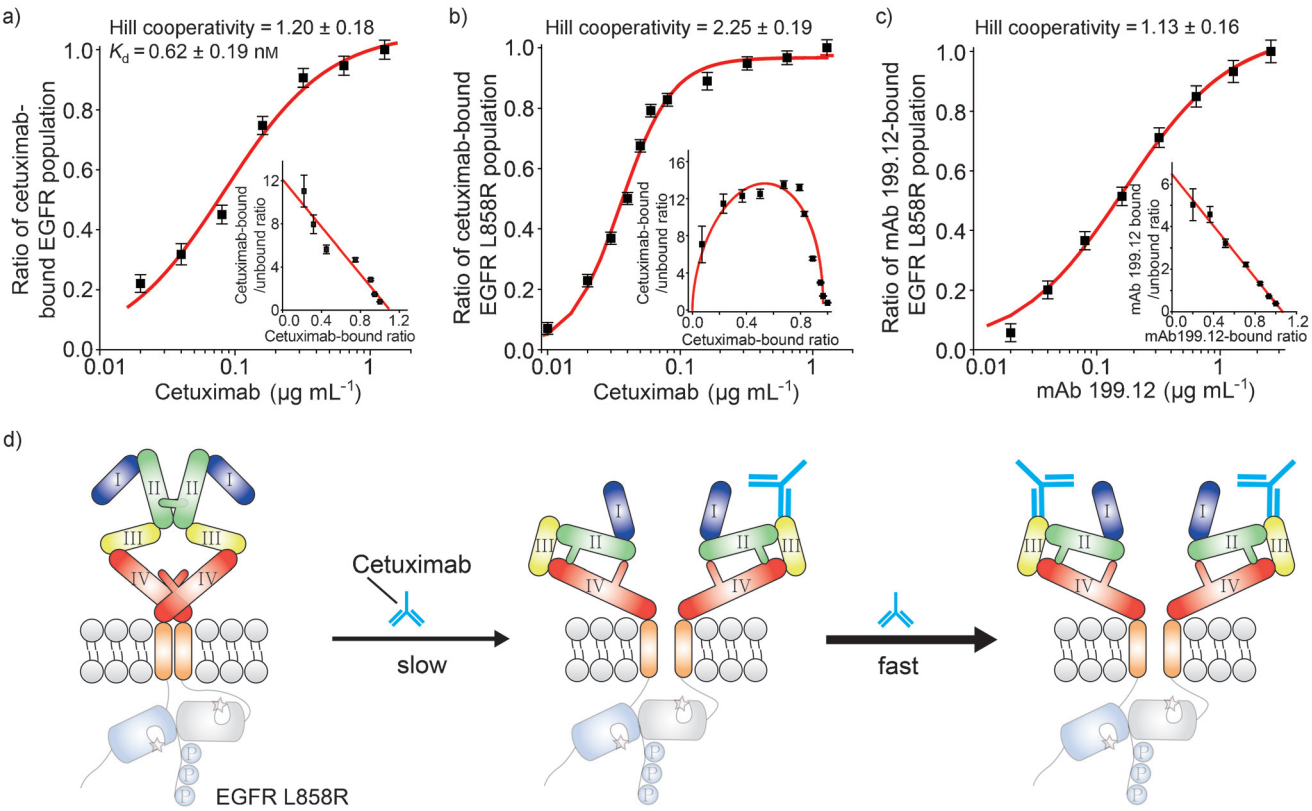

Figure 5. Dissociation constant and cooperativity measurement for the interaction of cetuximab with EGFR and its mutants. a) Analysis of the dissociation constant and cooperativity of cetuximab binding to EGFR on a COS7 cell membrane. b,c) Analysis of the dissociation constant and the cooperativity of the binding of cetuximab (b) and mAb 199.12 (c) to EGFR L858R on a COS7 cell membrane. The insets show Scatchard plots to evaluate the interaction cooperativity. The error bar represents the s.e.m. of a single-molecule population ($n > 5000$). d) Proposed model for the molecular mechanism of the positive cooperativity of cetuximab binding to EGFR L858R.

mAb 199.12,[16] these results suggest that EGFR L858R exists as a dimer with an extended ECD conformation. Thus, the binding of a first cetuximab molecule to one of the extended extracellular domains III, although unfavorable, facilitates the binding of a second cetuximab molecule by converting the remained cetuximab-free ECD into the preferable tethered conformation.[17] This conversion most likely involves the loss of the dimerization-arm interaction that maintains the stability of the dimerized extended ECDs.[18] The lack of cooperativity of cetuximab binding to EGFRvIII, which has been reported to form a dimer but does not have a dimerization arm[19] (see Figure S18), is consistent with this model (Figure 5d).

Herein, we presented smDIMSA, which enables the investigation of membrane-protein interactions with soluble ligands in the membrane of living cells on the basis of the diffusivity of membrane proteins. The superior sensitivity of smDIMSA as a result of single-particle tracking with super-resolution microscopy techniques enabled us to detect a small change in the diffusion coefficient of a membrane protein upon the direct binding of the Fab fragments of antibodies.

Exploitation of the intrinsic diffusivity of membrane proteins in smDIMSA makes it possible to detect a ligand–receptor interaction without labeling the ligand. In contrast to the use of surface plasmon resonance for measuring a ligand–receptor interaction, which requires labor-intensive membrane-protein purification and expensive gold surfaces,[20] smDIMSA directly measures a ligand–receptor interaction on a single live cell seeded on an inexpensive glass surface, thus greatly reducing experimental labor and cost as well as the artifacts derived from the purification of intact membrane proteins for such in vitro assays.[21] Furthermore, smDIMSA can be used to investigate the interactions of endogenous membrane proteins by using photoswitchable organic dyes for dSTORM[22] (see Figure S19).

It remains a puzzle how the soluble region can affect the diffusivity of a membrane protein, given that the viscosity of aqueous media is orders of magnitude lower than that of a cell membrane. Recent studies have demonstrated the hop diffusion of a membrane protein in the membrane of living cells as a result of the submicron-scale partitioning of a cell membrane by membrane skeletons and immobilized picket proteins.[23] It may be possible that the picket proteins, which contain large ECDs, reduce the hop rates of EGFR diffusion owing to the increased hydrodynamic radius of the ECD of EGFR as a result of the binding of a soluble ligand, which consequently decreases the macroscopic diffusion coefficient of the membrane protein.

Keywords: cell membranes · diffusion · ligand–receptor interactions · super-resolution microscopy · transmembrane proteins

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