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Review Article (Invited)

Triple-color single-molecule imaging for analysis of the role of receptor oligomers in signal transduction

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Membrane receptors provide interfaces of various extracellular stimuli to transduce the signal into the cell. Receptors are required to possess such conflicting properties as high sensitivity and noise reduction for the cell to keep its homeostasis and appropriate responses. To understand the mechanisms by which these functions are achieved, single-molecule monitoring of the motilities of receptors and signaling molecules on the plasma membrane is one of the most direct approaches. This review article introduces several recent single-molecule imaging studies of receptors, including the author's recent work on triple-color single-molecule imaging of G protein-coupled receptors. Based on these researches, advantages and perspectives of the single-molecule imaging approach to solving the mechanisms of receptor functions are illustrated.

◀ *Significance* ▶

Cells sense a variety of extracellular signals and respond appropriately. The interfaces for sensing extracellular signals are membrane receptor molecules. Receptors have mechanisms to sense their cognate signals with high sensitivity while eliminating noise. A most direct approach to investigate the mechanisms of the conflicting functions of receptors is single-molecule observation in living cells. Recent advances in statistical and mathematical methods realize detailed motility analysis in single molecule imaging. In this review, potential of single-molecule imaging for solving problems in signal transduction mechanisms is illustrated through recent several works on single-molecule live-cell imaging.

Introduction

  Membrane receptors are essential for biological investigations from basic sciences in cell and molecular biology and applied sciences such as drug discovery. The receptors sense extracellular stimuli and initiate intracellular signaling events by transmitting the signal to downstream intracellular signaling proteins. Downregulation of the signaling will prevent appropriate cellular processes, whereas overactivation of the receptor function often causes such diseases as tumorigenesis. Identification of signaling networks and molecules involved in the network have been performed intensively through biochemical and molecular biological analysis, and now blueprints on complex signaling networks are available [1]. On the other hand, it is still obscure how the molecules in the network function in living cells. The dynamics of the molecules of interest in living cells will provide essential clues to solve the problem. Single-molecule imaging is one of the most direct approaches to obtaining information on the target molecule dynamics in living cells [2]. This review introduces examples of single-molecule imaging studies, mainly the triple-color single-molecule imaging of FPR1 [3], a species of G protein-coupled receptors (GPCRs), and overviews prospects of the single-molecule imaging. This review article is an

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extended version of the Japanese article [4].

Single-Molecule Imaging of Signaling Molecules on the Plasma Membrane

Single-molecule imaging is a unique technique among fluorescence imaging methods, which helps obtain information on the unaveraged behavior of individual molecules in real-time [2,5]. This feature of single-molecule imaging realizes monitoring such state of individual molecules as oligomerization [6] and motility [7] in addition to phenomena occurring in short-time and small numbers of molecules [8] hidden in time and number averaging in ensemble imaging methods. In other words, single-molecule imaging is a powerful tool for analyzing the formation and dissociation of each short-term molecular interaction, detecting molecular states that are the minority in number, and identifying the clustering and complex formation of individual molecules.

Single-molecule imaging, however, has several limitations in comparison to other live-cell imaging techniques. Single-molecule imaging usually requires intense excitation light and, therefore, is suffered by rapid photobleaching. Single-molecule spots detection is difficult in the high-density condition of the target molecules. Moreover, single-molecule fluorescence intensity severely fluctuates, making it difficult to estimate the number of molecules included in a single fluorescent spot with accuracy. These limitations often restrict the experimental conditions to perform analysis of single-molecule motility and assembly states in living cells.

For single-molecule live imaging, total internal reflection fluorescence microscopy is often adopted because it reduces background light, which helps detect weak single-molecule fluorescent spots [9]. Since evanescent light produced by total internal reflection illumination can reach just the vicinity of the basal plasma membrane of the sample cell, molecules in and on the plasma membrane like receptors are suitable targets for single-molecule imaging studies. Various findings have been obtained on the mechanism of extracellular signal transduction through receptor molecules based on the analysis of receptor motility [10-12].

Membrane receptors are generally trans-membrane proteins that transport extracellular stimuli into the cell. When a ligand binds to the extracellular domain of a receptor molecule in the plasma membrane, the intracellular domain interacts with cytoplasmic signaling proteins to transmit the signal into the cell. Notably, a simple model of receptor function based on one-to-one binding of a ligand and a receptor is not sufficient to explain the mechanisms of the properties required to receptors, such as a wide dynamic range or an acute response in signaling activity with a threshold at a specific ligand concentration, and a combination of high sensitivity and low noise [13]. Various factors are known to regulate receptor function, such as the dimer or oligomer formation of receptors and related proteins [14,15], the dependence of lipids in the plasma membrane [16], and actin meshwork located just below the plasma membrane [17]. However, the detailed regulation mechanisms of receptor function are not fully understood yet.

Since the realization of single-molecule fluorescence imaging in living cells, several studies have been reported on the mechanism of receptors and signal transduction through single-molecule live imaging. A prominent single-molecule imaging study of membrane protein and signal transduction in living cells was conducted on a GPI-anchored protein CD59 [16]. In this study, the single-molecule diffusion motility of CD59 on the plasma membrane was tracked and analyzed in the monomer and crosslinked states, respectively. The analysis results showed that the sub-second temporary arrest of lateral diffusion, named STALL, increased by crosslinking of CD59. The dual-color single-molecule imaging of CD59 with downstream molecules Gαi2 or Lyn showed that the STALL was initiated by colocalization of CD59 and Gαi2. Then Lyn colocalized to the CD59 cluster during the STALL. These observation results suggest that a single CD59 cluster introduces a pulsed signal into the cell that is shorter than one second, which is much shorter than the duration time of the cellular signaling response. These findings were made possible by single-molecule live-cell and demonstrated the potential of single-molecule imaging to reveal the mechanism of signal transduction.

In another example, single-molecule monitoring of epidermal growth factor receptor (EGFR) was reported.[18] In this report, the variable Bayes-hidden Markov model (VB-HMM) was used to analyze the motility of and clustering states in single EGFR trajectories before and 15, 30, 60 seconds after EGF stimulation. The motility the EGFR was categorized into three classes: immobile, slow mobile, and fast mobile. Before the stimulation, EGFR in the immobile and slow mobile modes showed confined diffusion motions, whereas the fast-mobile EGFR exhibited free diffusion on the plasma membrane. Interestingly, the confinement condition of the EGFR diffusion altered upon the EGF stimulation; the confinement of the slow-mobile EGFR dissolved in 15-30 seconds after the stimulation, and then the diffusion area was narrowed again to be smaller than the initial value at 60 seconds. This alteration of the confinement area may help accelerate the EGFR association to form dimers or clusters required for signaling reaction. Recently, the mechanism of Son of Sevenless (SOS) activation, which is an upstream molecule of the MAP kinase pathway, on the membrane was reported [19]. In this study, the dwell time of SOS localization on the artificial membrane was estimated by single-molecule imaging. The dwell time analysis of single SOS molecules exhibited 'activation lag time' in which SOS kept localizing on the membrane before activation. After the lag time, activated SOS then crosslinked a transmembrane protein LAT to induce phase separation, which provides a scaffold of Ras activation. The presence of the lag time would help to

proofread SOS activation. Such proofreading manner of SOS activation using the lag time and scaffold formation that would contribute acceleration and preciseness in signal transduction was revealed by an unaveraged motion of each SOS molecule on the membrane monitored by single-molecule imaging. Another investigation used single-molecule imaging to understand the wide dynamic range of membrane receptors. Miyanaga et al [20]. performed FRET analysis and single-molecule imaging of cAMP receptor (cAR1) and G proteins in *Dictyostelium discoideum* to understand the mechanism of its wide dynamic range. In the FRET analysis, dissociation of Gα-Gβγ was detected upon cAMP stimulation. Single-molecule imaging of Gα and cAR1 demonstrates that more than 50% of Gα showed the slower diffusion dynamics upon cAMP stimulation. The diffusion coefficient of the slower fraction was comparable to that of cAR1, the suggesting interaction of Gα and cAR1. Moreover, the localization duration time of Gα to the plasma membrane became longer under the administration of cAMP. While the EC50 values of cAMP concentration on the Gα-Gβγ dissociation were estimated to be 2.3 nM, the appearance of Gα fraction showing the slow diffusing and the longer membrane-localization duration has 12 nM and 270 nM of half maximum, respectively. This inconsistency of cAMP dependency in the Gα dynamics events may realize the wide dynamic range of the cell to respond to the stimulation of cAMP.

Thus, the single-molecule imaging technique elicits non-averaged motion and clustering states of the target membrane molecules. Such approaches through single-molecule imaging have been used to monitor and analyze receptor clustering on the plasma membrane. Dimerization and oligomerization of membrane receptors are involved in precise signal induction through the receptors. Understanding the detailed molecular mechanism and roles of receptor oligomerization requires direct monitoring of receptor dynamics on the plasma membrane in living cells. Some previous studies demonstrated single-molecule observation to analyze dimerization/oligomerization and signal transduction. Möller et al. performed single-molecule imaging of a GPCR, μ opioid receptor (μOR), to monitor its clustering after stimulation [21]. The motion of μOR exhibited rapid diffusion 1 min after agonist stimulation and then became slower than before stimulation. Oligomers of μOR were also generated along with the diffusion became slower after an agonist DAMGO stimulation that induces internalization with β-arrestin, whereas another agonist morphine, which does not induce internalization of μOR, did not lead the oligomer formation. Considering the similar time course of μOR oligomer formation and interaction β-arrestin after DAMGO stimulation, μOR large oligomer formation may be involved in complex formation with β-arrestin that results in endocytosis of μOR oligomers. Another GPCR, dopamine D2 receptor (D2R), was also investigated to analyze its dimerization [22]. Whereas Möller analyzed diffusion coefficient and interaction with β-arrestin of μOR, Kasai et al. performed the single-molecule analysis of D2R mainly focusing duration time of D2R transient dimer formation upon stimulation. Kasai and coworkers analyzed colocalization of D2R spots on the plasma membrane in living CHO cells based on single-molecule imaging. Based on the analysis, single-molecule spots of D2R colocalized with each other with the lifetime of 68 ms before stimulation, which elongated to 99 ms upon dopamine stimulation. Similar elongation of the duration time occurred upon another agonist, quinpirole, stimulation (104 ms), but the antagonist did not induce such duration time elongation. These results imply that the elongation of duration in the D2R dimer formation would involve the signaling event through D2R in the plasma membrane. In a recent study, Nishiguchi et al. investigated the role of oligomerization of a species of GPCRs, FPR1, in signal transduction from the GPCR to G proteins using triple-color single-molecule imaging in living cells [3]. In the following, this research is introduced in detail.

FPR1

GPCRs are a large family of proteins and the most significant drug discovery target. There are more than 800 species of GPCRs in mammalian, and about 30% of medical drugs target GPCRs [23,24]. The target molecule in the study was formyl peptide receptor 1 (FPR1), a GPCR existing prominently in immune cells such as phagocytic and leukocyte cells and functions as an immune receptor activated by N-formyl peptides [25-27]. While FPR1 plays an essential role in the immune response against bacteria, overactivation of this GPCR is involved in malignant tumor progression and severe drug eruption of the skin [28]. Thus, the activation and signal transduction of FPR1 should be strictly regulated in the cells, and the mechanism of signaling regulation is an important target for drug discovery. A single-molecule imaging approach was used in the investigation of the FPR1 activation mechanism especially addressed to analyze the role of oligomerization of FPR1 in the signal transduction [29].

FRET Assay System for FPR1 Signaling Estimation

Consecutive observation of single-molecule motion for tens of seconds is technically difficult because of the rapid photobleaching of fluorophore. Therefore, determining the time to perform a single-molecule observation after ligand stimulation is necessary before conducting single-molecule imaging. The time course of signal transduction was monitored after ligand stimulation using a FRET technique to estimate the appropriate time to conduct single-molecule FPR1 observation. In this FRET experiment, cyan fluorescent protein (CFP)-fused Gα and yellow fluorescent protein

(YFP)-fused Gγ were expressed in FPR1-expressing HEK293 cells. When FPR1 receives ligands and transmits the signal to G proteins, dissociation of the trimeric G is induced, and FRET from CFP to YFP disappears (Fig. 1). Stimulation of the cell sample with FPR1's ligand, fMLP, induced rapid FRET decay that was maximal 30 seconds after the stimulation. Considering this result and that Arrestin recruitment to FPR1, which prevents the signaling activity of G proteins, will occur after the G protein activation, the single-molecule observations were performed within 30 seconds after ligand stimulation in future experiments.

## Dual-Color Single-Molecule Imaging of FPR1 Oligomers and G proteins

Next, dual-color single-molecule imaging of FPR1 and G protein in living cells was performed to monitor oligomerization of FPR1 and the signal transduction from FPR1 to G proteins simultaneously in the same cells. Two

Figure 1 A Schematic of the FRET analysis. Gα and Gγ were fused with CFP and YFP, respectively. By activation of FPR1 upon ligand binding, the trimeric G protein dissociated, and FRET is resolved.

fusion proteins were prepared for this observation: FPR1 fused with a SNAPf-tag on its extracellular NH_2 terminus (FPR1-SNAPf), and Gα conjugated with a Halo-tag in its flexible region (Gα-Halo). The expression vectors of these fusion proteins were introduced into HEK293 cells. The produced SNAPf-FPR1 and Gα-Halo were then labeled with Setau647 and tetramethylrhodamine (TMR), respectively. These proteins showed the same localization features as endogenous FPR1 and Gα.

In single-molecule imaging to investigate the role of FPR1 oligomer formation in the signal transduction, the density of FPR1 should be optimized to be the same as the endogenous FPR1 in immune cells because the formation of oligomers is strongly affected by the concentration of the molecule. In this study, we established an FPR1 stably-expressing cell line in which the density of SNAPf-FPR1 in the plasma membrane was 2.1 molecules/μm^2;[29] the same density of FPR1 as in neutrophils in which FPR1 functions in the immune system. In this density of FPR1, however, fluorescent spots are often overlapping, and precise detection, tracking, and intensity analysis of the spots are difficult. In the single-molecule FPR1 observation experiments, the labeling efficiency was lowered to be about 20% to overcome the difficulty in detecting and tracking fluorescent spots. Under this labeling condition, each fluorescent spot potentially includes FPR1 molecules without labeling, which causes underestimation of the number of the FPR1 molecules in a single spot from the calculation based on the fluorescence intensity. Considering this difficulty in estimating the accurate number of FPR1 in the fluorescent spots, the FPR1 spots were roughly classified into two categories: small oligomers that showed the same fluorescence intensity as a single-fluorescent molecule and large oligomers that emitted stronger fluorescence than a single-fluorescent molecule.

In the single-molecule fluorescence movies, the small and large oligomers of FPR1 coexisted even before stimulation with the ligand fMLP. Upon stimulation with fMLP, the large oligomers fraction was increased after 10 seconds from the stimulation. This oligomer formation was much earlier than the GPCR-Arrestin complex formation that induces the generation of the clathrin-coated pit. In the dual-color imaging, the dwell time of FPR1—Gα colocalization is expected to be longer than that without signal transduction because the dissociation of trimeric G protein occurred on the FPR1 when the signal was transmitted from the FPR1 to the trimeric G proteins. Based on a hypothesis that the large oligomer formation involves activating signal transduction, the colocalization dwell time was analyzed and compared between the small and large oligomers before and after stimulation. Contrary to the expectations, no significant differences in the dwell time were detected between the large oligomers before and after the fMLP stimulation, even though the dwell time becomes slightly longer upon the stimulation.

Triple-Color Single-Molecule Imaging of FPR1, Ligand, and Gα

A potential reason why no significant difference was obtained in the dwell times of FPR1—Gα colocalization in the dual-color imaging is that ligand-bound and unbound FPR1 coexists even after fMLP addition. FPR1s in the ligand-bound and unbound states cannot be distinguished in the dual-color single-molecule imaging of FPR1 and Gα. To distinguish ligand-bound and unbound FPR1 in single-molecule imaging, the ligand molecule was fluorescently labeled and observed simultaneously with FPR1 and Gα. A TMR-labeled FPR1 ligand, fNLFNYK-TMR, was prepared to perform this observation. SNAPf-FPR1 and Gα-Halo were stained with Setau647 and R110, respectively (Fig. 2A). Triple-color
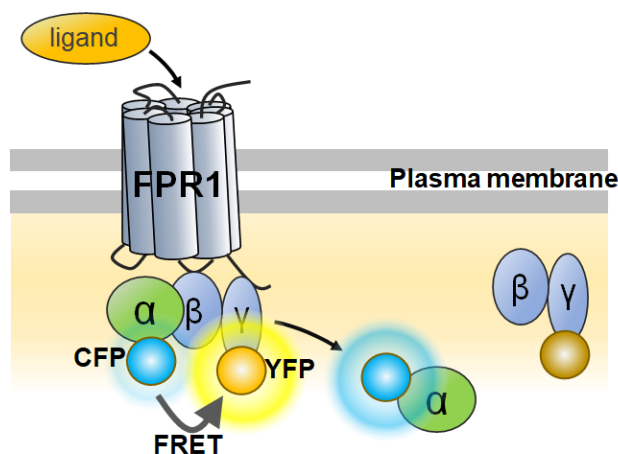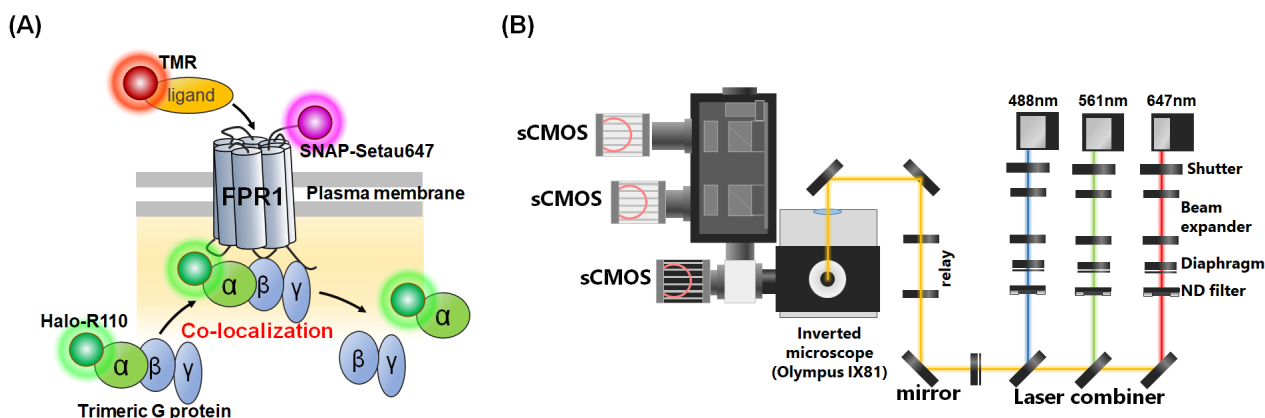
Figure 2 (A) a schematic of the observed molecules in the triple-color single-molecule imaging [4]. (B) microscope setup for the triple-color single-molecule imaging.
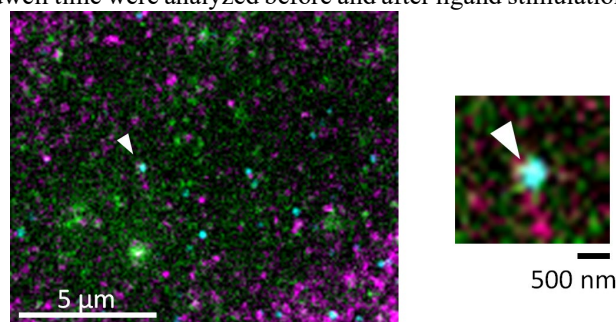
single-molecule observation of ligand, FPR1, and Gα was conducted using these molecules under a TIRF microscope equipped with multiple excitation laser lines and three scientific complementary metal-oxide semiconductor (sCMOS) cameras to acquire the fluorescence signals of each of the three colors (Fig. 2B) [30,31]. In the observation, the laser lights of 488, 561, and 647 nm were introduced to the sample as a TIRF illumination to the sample as excite R110, TMR, and Setau647, respectively. The emitted fluorescence was separated through dichroic mirrors and bandpass filters to green, red, and deep red fluorescence lights and introduced to each sCMOS camera. The captured images were overlayed using a method previously reported by Koyama-Honda et al [32]. The precisions of localization and superimposition of the fluorescent spots were about 30 nm and 35 nm respectively.

In this triple-color observation (Fig. 3), FPR1 spots were categorized into four classes: ligand-unbound small oligomer, ligand-bound small oligomer, ligand-unbound large oligomer, and ligand-bound large oligomer (still noteworthy that the ligand-unbound oligomers possibly include ligand-bound oligomers in which the fluorescence-labeled ligand is invisible because of decomposition of the fluorophore by photobleaching, etc.). Through the triple-color single-molecule imaging, FPR1 oligomer formation and the FPR1—Gα colocalization dwell time were analyzed before and after ligand stimulation.

Firstly, the stimulation dependency on the FPR1 oligomer formation was estimated by analyzing fluorescence intensity on the individual spots. In this analysis, the large-oligomers were significantly increased in the ligand-bound FPR1 spots compared to the ligand-unbound spots. Moreover, the FPR1—Gα colocalization dwell time was analyzed on the four classes of FPR1 categorized based on the ligand binding states and oligomer sizes. In the analysis results, whereas no significant dwell time difference was detected between small and large oligomers in the ligand-unbound state, the dwell time became significantly longer on ligand-bound large oligomers than ligand-bound small oligomers. These results indicated that intense signal transduction to G proteins occurred on the ligand-bound large oligomer FPR1 compared to other classes of FPR1 spots.
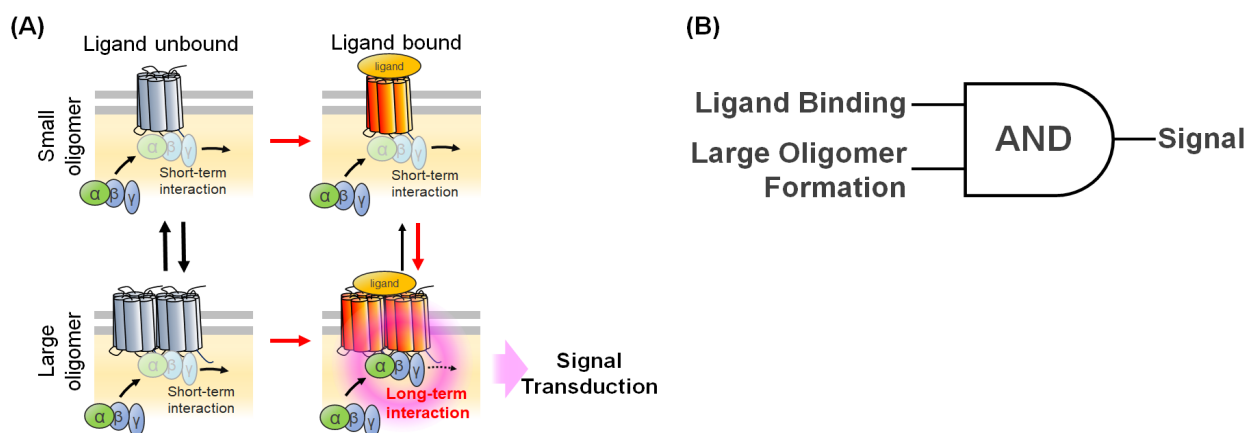
Summary and Future Perspective

Figure 3 A snapshot of triple-color single molecule imaging of FPR1, fNLFNYK-TMR, and Gα. Cyan, magenta, and green spots represent SNAPf-FPR1 (Setau647), fNLFNYK-TMR, and Gα-Halo (R110), respectively [4]. The arrowheads represent the spots of triple-color colocalization. The right panel is an enlarged view of the colocalization spot.

In the research introduced here, analyses of ligand-binding states and colocalization dwell time of FPR1 and G proteins were performed through triple-color single-molecule imaging. From the single-molecule observations, ligand-bound large FPR1 oligomers are suggested to be a highly active form for the signal transduction compared with the other ligand binding and oligomerization forms (Fig. 4A). This result implies that FPR1 functions as an AND logic gate for the signal transduction; simultaneous two inputs, ligand binding and large oligomer formation, trigger the significant signal output to the G proteins (Fig. 4B). This mechanism may help achieve both quick response to the extracellular stimulations and highly accurate noise reduction. The single-molecule observation showed that large FPR1 oligomers existed even before the ligand stimulation. If the large oligomers introduce the signal into the cell immediately after their formation, noise signals are always introduced independently of ligand stimulation. On the other hand, if the large oligomer forms only

Figure 4 Summary of the results of triple-color single-molecule imaging of FPR1, Gα and the ligand. (A) schematic of relationship among oligomer size, ligand binding state, and signaling efficacy [4]. (B) Logic gate of FPR1. Activation of FPR1 requires both ligand binding and large oligomer formation, which means that FPR1 functions as AND logic gate.

after ligand binding to FPR1, the response to the ligand will be delayed because FPR1 collision to form large oligomers will take time. Thus, basal equilibration of small and large oligomers and the AND logic gate system would help the precise signal transduction through FPR1.

This work purposely lowered labeling efficiency to create observation conditions that allow separately detecting each fluorescent spot. In this condition, however, accurate estimation of the number of FPR1 in each fluorescent spot is difficult, and therefore, the FPR1 spots were roughly categorized into small oligomers and large oligomers. Recently, some methods have been reported to perform fluorescent spot detection even in dense conditions [33,34]. These methods may be useful for more accurate spot detection and estimation of the number of target molecules in each spot in sample conditions closer to the original expression level.

Thus, techniques in single-molecule imaging in living cells have recently been developed to provide clues to understand the function and mechanisms of the target molecules, and moreover may have an ability to contribute applied sciences. The recent development of a high-throughput single-molecule imaging system would contribute to single-molecule imaging to translational and applied chemistry such as drug discovery [35,36]. In addition to the ability of high-throughput imaging, estimation of activity of the target molecule from its motility is required for the application of single-molecule imaging to drug discovery. Yanagawa et al. performed an analysis of diffusion motion of a class C GPCR, metabolic glutamate receptor 3 (mGluR3), under various ligand conditions through single-molecule live-cell imaging [37]. In this work, the single-molecule imaging analysis demonstrated increases and decreases in the average diffusion coefficient of mGluR3 upon ligand-dependent inactivation and activation. Moreover, the same single-molecule imaging analysis was conducted on a variety of class A and B GPCRs and found that agonists induced decreasing the average diffusion coefficient of the GPCRs. These results imply that averaged diffusion coefficient of membrane receptors would have the potential to provide a good index to evaluate drug efficacy in the drug discovery field. The targets of single-molecule live-cell imaging have been expanding beyond molecules on the plasma membrane. For example, single-molecule imaging-based observation was performed on cluster formation of chromatin structure components. RNA, in addition to protein, is becoming a target for single-molecule imaging in living cells due to the development of fluorescent labeling methods on endogenous RNAs in living cells [12,38-41]. Moreover, a technology to overcome the limitation of observation time due to the photobleaching was developed, and single-molecule motions during target cellular events were now possible to be tracked continuously over several minutes [42]. These techniques will be helpful to apply a single-molecule imaging approach to various fields of basic and applied biology.

The single-molecule imaging approach would be compatible with mathematical modeling and simulation because parameters provided from single-molecule analysis, such as positional coordinates, frequency of colocalization, colocalization lifetime, etc., can be used to establish mathematical models and simulation. These are useful for assembling mathematical models and simulations to solve the mechanism of physiological events. For example, a mathematical modeling study of receptor assembly, ligand binding, and G protein binding based on the FPR1 single-molecule imaging study has been reported [43]. Such mathematical modeling and simulation of molecular motion based on single-molecule analysis would be a powerful approach to solving cellular physiological events' mechanisms based on the view from molecular motions.

Conflict of Interest

Author Contributions

Acknowledgements

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