Raftophilic rhodopsin-clusters offer stochastic platforms for G protein signalling in retinal discs

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Rhodopsin is a G protein-coupled receptor (GPCR) that initiates the phototransduction cascade in retinal disc membrane. Recent studies have suggested that rhodopsin forms highly ordered rows of dimers responsible for single-photon detection by rod photoreceptors. Dimerization is also known to confer to rhodopsin a high affinity for ordered lipids (raftophilicity). However, the role of rhodopsin organization and its raftophilicity in phototransduction remains obscure, owing to the lack of direct observation of rhodopsin dynamics and distribution in native discs. Here, we explore the single-molecule and semi-multimolecule behaviour of rhodopsin in native discs. Rhodopsin forms transient meso-scale clusters, even in darkness, which are loosely confined to the disc centre. Cognate G protein transducin co-distributes with rhodopsin, and exhibits lateral translocation to the disc periphery upon activation. We demonstrate that rhodopsin offers inherently distributed and stochastic platforms for G protein signalling by self-organizing raftophilic clusters, which continually repeat generation/extinction in the disc membrane.
G-protein-coupled receptors (GPCRs) represent the third largest family of genes in the human genome. Extensive studies have been carried out on the structure and function of GPCRs, and now have been extended to investigations into the functional significance of their dimerization or higher oligomerization1–3. Oligomerization of GPCRs has the potential to affect all aspects of the signaling cycle, including receptor biogenesis, activation and desensitization1. Furthermore, attention has been drawn to the membrane-mediated oligomerization and related compartmentalization of GPCRs into membrane nanodomains, i.e. rafts or caveolae, and to the implications of such nanodomains for GPCR functions2. The nanodomains are characterized by their "raftophilicity"4, i.e. a favourability to ordered lipids in the liquid-ordered (Lo) state, and segregated from the more loosely organized bulk lipid bilayer in the liquid-disordered (Ld) state.

Vertebrate phototransduction machinery is a prototypical G-protein-signalling system, extensively studied and thought to be fully understood. In the classical scenario, the signalling process is explained by the diffusion-collision coupling between key players, i.e. the photopigment rhodopsin, cognate trimeric G protein transducin (Gt) and its target enzyme 3’,5’-cyclic guanosine monophosphate-phosphodiesterase (PDE6)5. Photoisomerized rhodopsin (Rh*) binds to Gt, to form a 2:1 complex (Rh*2–Gt complex)7. In the absence of guanosine-5’-triphosphate (GTP), Rh* catalytically activates ~10 Gt molecules within the lifetime of Rh*9,10. Following nucleotide-exchange on the α-subunit of Gt (Gα), activated Gα starts to diffuse across the membrane to activate its target enzyme, PDE6. The rhodopsin/Gt/PDE6 ratio in the disc membrane is set at approximately 100:10:1 to 111. The cGMP-hydrolysis results in hyperpolarization of the plasma membrane by closing cyclic nucleotide-gated ion channels. These processes have been explained by the diffusion-based coupling of membrane proteins11. However, the theory regarding the physical background upon which these processes occur has recently been challenged. Atomic force microscopy (AFM) revealed a paracrystalline arrangement of rhodopsin dimers12, suggesting a lower degree of lateral diffusion of rhodopsin in the disc. Subsequent AFM studies have shown meso-sized "nanodomains" without internal structure, which are loosely confined in the central area of the disc membrane13–15. A cryo-electron tomographic study has shown that at least ten rhodopsin dimers form pairs of rows (tracks) aligned parallel to the disc incises16, and their accompanying simulation results suggested that the track structure can explain the uniform single-photon response in rod photoreceptors, a long-standing question in phototransduction studies16. Although such structural studies have provided static pictures of the supramolecular structure of rhodopsin, a coarse-grained molecular-dynamics simulation study implied that the rhodopsin organization would be formed through relatively weak (1.2–3.6 kcal/mol) protein–protein interactions, via multiple dimerization interfaces, and that the organization should be transient17. In addition, accumulated evidence indicating that the average diffusion coefficient of rhodopsin is in the range of 0.1–0.6 μm² s⁻¹18–24 strongly suggests that rhodopsin molecules are diffusive in the disc. Moreover, we should note that rhodopsin is expected to be in dynamic equilibrium between monomers, dimers, and higher-order oligomers25–27 and that the transition between these states is regulated by the balance of protein–lipid, protein–protein, and lipid–lipid interactions25.

The disc membrane is also known to have another type of inhomogeneity, presumably based on the raftophilicity of oligomerized-rhodopsin. We and others have already found that 10–30% of rhodopsin in the dark-adapted disc membrane is recovered in the detergent-resistant membrane (DRM)28,29, the distribution of which is quite a useful index of raftophilicity for membrane proteins30. Furthermore, Gt exhibits noticeable translocation to DRM from detergent-soluble membrane when rhodopsin is isomerized28, suggesting that Rh* activates Gt in the raftophilic membrane domain in the disc. These results suggest that there are rhodopsin-containing raftophilic membrane domains in the disc membrane, where Rh* activates Gt. Our subsequent study revealed that the Gt-stabilized dimer of rhodopsin is responsible for the raftophilicity of the Rh*–Gt complex, and that palmitoyl modification of rhodopsin is a prerequisite for raftophilicity attained by dimerization31.

To explore such raftophilicity-based membrane inhomogeneity, previous studies on the raft of the plasma membrane provide important clues32,33. The "raft" has been defined as a molecular complex consisting of at least three molecules, including a molecule with a saturated alky chain or a cholesterol molecule that plays a critical role in the creation of the complex itself2. The raft in the plasma membrane of an unstimulated cell is generally short-lived and nano-sized34. However, when transmembrane proteins having raftophilic moiety (e.g., glycosylphosphatidylinositol-anchored receptor protein (GPI-AR)) are oligomerized with ligand or immunoglobulin G (IgG), they form meso-sized, long-lived raftophilic receptor-clusters, the so-called 'receptor-cluster rafts'4, in which raftophilic lipids and proteins are recruited to stabilize the cluster raft. Here we should note that rhodopsin, like GPI-AR, has two saturated alkyl chains (di-palmitoyl)32, which provides a strong raft-targeting mechanism for transmembrane proteins33. Despite palmitoylation, monomeric rhodopsin is non-raftophilic (raftophobic) and excluded from the Lo-phase in a cholesterol-dependent manner34. On the contrary, like GPI-AR, rhodopsin attains a high raftophilicity upon stabilized-dimerization35. Therefore, the very close resemblance between GPI-AR and rhodopsin leads us to hypothesize that rhodopsin forms a receptor-cluster raft when Gt or IgG stabilizes a rhodopsin dimer. Furthermore, the presence of a considerable population of rhodopsin in the DRM of dark-adapted discs also implies that rhodopsin spontaneously forms receptor-cluster rafts even in darkness.

Here we aim to confirm the presence of dynamic inhomogeneity due to transient formation of rhodopsin-cluster rafts in native disc membrane. We explore the single-molecule behaviour of rhodopsin and transducin in native disc membrane by single-molecule tracking with a near-infrared (near-IR) wavelength. Applying variational Bayes hidden Markov model (HMM) analysis35,36 to the single-molecule diffusion of rhodopsin, we derive the number of diffusion states of rhodopsin and the transition rates between them, and examine the effect of light, GTP and cholesterol-depletion on them. In addition, we explore the collective behaviour of rhodopsin and other membrane molecules using the "semi-multimolecule fluorescence imaging" technique that we have developed. Our results clearly show that rhodopsin forms transient meso-sized raftophilic clusters, loosely confined in the disc membrane, which are excluded from the raftophobic disc periphery. The stochastic nature of the physical background for phototransduction is revealed.

Results

Single-molecule tracking study of rhodopsin in frog native discs. We first investigated whether rhodopsin forms dynamic clusters in dark-adapted native disc membranes of bullfrog (Rana catesbeiana), by performing single-molecule tracking of rhodopsin and transducin on a total internal fluorescence microscope (TIRFM) using a near-IR wavelength. Assuming that dynamic clustering transiently retards the diffusion of rhodopsin, we subjected the single-molecule tracking data to variational Bayes hidden Markov model (HMM) analysis33, to infer the number of diffusive states and transition rates between them36.
As the specimen, we used disc membranes (~8 µm in diameter) exposed at the end of mechanically fragmented rod outer segments (f-ROSs) from frog rod photoreceptors (Fig. 1a). Rhodopsin was probed with the Fab′ fragment of the anti-rhodopsin monoclonal antibody 1D4 (Fab′-1D4) (Supplementary Fig. 1a) labelled with a near-IR dye (Fig. 1b). Fluorescently labelled Fab′-1D4 on the disc membrane was illuminated with a highly inclined laser beam (750-nm in wavelength), almost parallel to the glass surface on a TIRFM (Fig. 1c), and single-molecule fluorescent spots were observed (Fig. 1d). This approach matches that called variable-angle evanescent microscopy37 or ‘pseudo-TIRF’ (Fig. 1c and Supplementary Fig. 2a).

Our first finding was that all fluorescently labelled rhodopsin molecules were mobile (Fig. 1e and Supplementary Movie 1). If the disc membrane contacts the glass surface, lateral diffusion of membrane proteins should be hindered. However, we found that there is a gap of about 280 nm between the bottom of the f-ROS and the glass surface (Supplementary Fig. 2a). The gap is likely filled with cytoplasm leached from the cut end of the f-ROS. Furthermore, it is highly likely that what we saw were fluorescent probes bound to the most-accessible disc surface, exposed at the bottom of f-ROS, since we did not see fluorescent spots when we moved the focus up above ~40 nm (almost one increment on fine-focus dial, corresponding to 1–2 disc stacks) from the bottom-disc surface (Supplementary Fig. 2a). In addition, the intensity distribution of fluorescent spots was almost a single Gaussian (Supplementary Fig. 2b), eliminating the possibility of bright spot agglutination due to the contact of disc with the glass surface.

To determine the effective microscopic lateral diffusion coefficient of rhodopsin in the disc, we used mean square displacement (MSD)-time interval plot analysis39. The effective microscopic diffusion coefficients of rhodopsin in an interval of 100 ms (D_{100 ms}) produced a broad single-peak histogram (Fig. 1f). The median value was in good agreement with previously measured macroscopic rhodopsin diffusion coefficients in discs18–24. The open-source software for variational Bayes single-particle tracking, i.e. vbSPT36 indicated a three-state optimal HMM for rhodopsin diffusion in dark-adapted discs (Fig. 1g; the complete results of the vbSPT analyses are given in Supplementary Fig. 3; the statistical credibility of the inference on the three-state HMM of Fig. 1g is shown in Supplementary Table 1). Trajectories can be colour-coded on the basis of diffusive state (Fig. 1h). The three diffusive states of rhodopsin are shown in the histogram of D_{100 ms} (Fig. 1i). There seemingly was a contradiction between the high occupancy of fast diffusive state-3 and the modest distribution of D_{100 ms} in the corresponding highly diffusive range. That contradiction is due to the conceptual difference between the diffusion coefficients calculated by MSD analysis and by vbSPT analysis. Whereas MSD analysis relies on time-series analysis, vbSPT analysis relies on time-series analysis using memory-less jumps between diffusive states. The former gives the time-averaged diffusion coefficient of a molecule that undergoes diffusion by transitioning through multiple diffusive states, whereas the latter gives the inherent diffusion coefficient of each diffusive state.

**Effect of light and GTP on rhodopsin diffusivity**

Next we examined the effect of light and GTP on the HMM of rhodopsin. Under all tested conditions, the optimal HMM had three diffusive states ostensibly exhibiting invariant diffusion coefficients (Fig. 2a, b). In the presence of GTP, photoisomerization of 20% of the rhodopsin caused no alteration in the HMM (Fig. 2a, b). However, in the absence of GTP, light increased the occupancy of highly diffusive range. That contradiction is due to the conceptual difference between the diffusion coefficients calculated by MSD analysis and by vbSPT analysis. Whereas MSD analysis relies on time-series analysis, vbSPT analysis relies on time-series analysis assuming memory-less jumps between diffusive states. The former gives the time-averaged diffusion coefficient of a molecule that undergoes diffusion by transitioning through multiple diffusive states, whereas the latter gives the inherent diffusion coefficient of each diffusive state.
of rhodopsin, in the absence of GTP, was confirmed by depletion and replenishment of G_{i} and/or cholesterol in dark-adapted disc membrane (Supplementary Fig. 4). On the other hand, vSPT results revealed that the light-dependent hindrance of rhodopsin diffusion is brought about by the increased occupancy of diffusive state-1 (Fig. 2d), by increasing its dwell time (Supplementary Fig. 3). This result suggests that the light-dependently formed Rh^{*}_{2}–G_{i} can organize a 'receptor-cluster raft'. In contrast, MCD did not affect the HMM of rhodopsin in dark-adapted discs, i.e. the slowest diffusive state (state-1) in HMM showed robust tolerance to cholesterol-depletion. Thus, it is highly likely that, even in darkness, rhodopsin molecules autonomously form stable rhodopsin-cluster rafts through protein–protein interactions in highly crowded discs. This presumably happens with the assistance of raftophilic disc lipids other than cholesterol, i.e. the saturated phospholipids such as di-C16:0-phosphatidylcholine in the discs.

Effect of cholesterol-depletion on HMM of rhodopsin diffusion. We previously found that the binding of G_{i} to Rh* stabilizes the rhodopsin dimer to make a highly raftophilic Rh^{*}_{2}–G_{i} complex\(^{8}\). Thus, it is tempting to speculate that Rh^{*}_{2}–G_{i} works like a condensation nucleus in the metastable disc membrane, cooperatively condensing raftophilic metastable rhodopsin dimers, cholesterol and saturated phospholipids. To test this assumption, we investigated how cholesterol participates in the diffusion of rhodopsin in the disc. That was accomplished by examining the effect of cholesterol-depletion, with methyl-β-cyclodextrin (MCD), on the optimal HMM of rhodopsin in dark-adapted or light-exposed discs (Fig. 2e). First, we found that the light-dependently increased state-1 occupancy seen in the absence of GTP can be restored to the level in the dark using 20 mM MCD.

Effect of stabilized-dimerization on rhodopsin diffusivity. To corroborate our assumption that the stabilized-rhodopsin dimer works like a condensation nucleus for rhodopsin-cluster rafts, even in darkness, we examined the diffusivity of IgG-crosslinked rhodopsin (Fig. 2f), which is known to be highly raftophilic\(^{8}\). The IgG-crosslinked rhodopsin showed an almost identical three-state HMM to that of rhodopsin when it is light-dependently hindered (compare Fig. 2g upper with 2a lower-middle panel). Cholesterol-depletion restored the HMM to the state-2-dominant one, very much like that in darkness (Fig. 2g lower and 2h).
The diffusivity of $G_{10}$, $Rh_{G}^{2}$–$G_{10}$, and $G_{10}$. The slow diffusion of the $Rh_{G}^{2}$–$G_{10}$ complex was also confirmed by using fluorescently labelled $G_{10}$ intact in its light- and GTP-dependent activation (Fig. 2i and Supplementary Fig. 1). The $D_{100\text{ms}}$ histogram of $G_{10}$ in darkness was almost identical to that of rhodopsin, and showed the light-dependent decrease and activation-dependent increase of $G_{10}$ diffusivity (Fig. 2j), in good agreement with a macroscopic study. Furthermore, the optimal HMM of $G_{10}$, in darkness (in the form of $G_{11}$) was a three-state model very similar to that of rhodopsin, irrespective of the presence or absence of GTP (Fig. 2i upper and 2k), suggesting pre-coupling of $G_{11}$ with dark-state rhodopsin. In the absence of GTP, photoisomerization of 20% of rhodopsin reduced the magnitude higher concentration of rhodopsin, irrespective of the presence or absence of GTP (Fig. 2j lower and 2k). The vbSPT did not generate any steady HMM for the activated-$G_{10}$, presumably owing to the complicated fate of activated-$G_{10}$, and its rapid dissociation from the disc membrane.

Collective behaviour of rhodopsin in dark-adapted discs. To further confirm the formation of rhodopsin-cluster rafts, we attempted to observe rhodopsin-clusters in dark-adapted disc membrane using semi-multimolecule fluorescence imaging. For that, we probed rhodopsin with an approximately two-orders-of-magnitude higher concentration of fluorescently labelled Fab′-1D4 (Fig. 3). Surprisingly, this imaging allowed us to observe apparently uneven (Fig. 3a), vigorously indentations (Fig. 3b), suggesting that rhodopsin-cluster rafts are loosely condensed in the central region of dark-adapted disc clusters and estimated diffusion coefficients of such clusters were in good agreement with those of diffusive state-1 in the HMM of rhodopsin in darkness (Fig. 3e, f), confirming that the diffusive state-1 is the transient cluster of rhodopsin.

Involvement of cholesterol in disc inhomogeneity. To explore the involvement of cholesterol in the clustering and distribution of rhodopsin in dark-adapted discs, we tested the effect of cholesterol-depletion. Cholesterol-depletion by 20 mM MCD flattened the distribution of rhodopsin-clusters but did not inhibit clustering itself (Fig. 4a). IgG-crosslinked rhodopsin, which is characterized by is high raftophilicity, showed cholesterol-dependent central confinement (Fig. 4b), corroborating our premise that rhodopsin-cluster rafts are segregated into the disc central area through cholesterol-mediated raftophilicity-based interactions.

Collective behaviour of $G_{10}$ in dark-adapted discs. We also confirmed the clustering of rhodopsin molecules, which are presumably pre-associated with $G_{10}$ in dark-adapted discs, by semi-multimolecule fluorescence imaging of fluorescently labelled $G_{10}$ (Fig. 5). The $G_{10}$ exhibited central confinement and transient clustering almost exactly like rhodopsin, corroborating our hypothesis. It is also interesting that an aggregate of fluorescently labelled $G_{10}$ was often observed at a point on the disc periphery, which is presumably ascribable to the axoneme.

Concentric heterogeneity in disc membrane. Our results above implied that disc membranes are segregated into two concentric regions, i.e. a raftophilic centre with confined rhodopsin-clusters,

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**Fig. 3** Collective behaviour of rhodopsin in disc membrane. a Representative snapshot of fluorescence imaging of a dark-adapted disc incubated with 26 nM fluorescent Fab′-1D4. Scale bar: 2 μm. b Fluorescence intensity profiles along the transverse line on a disc membrane and on the dashed line in a representative snapshot of single fluorescent spots of Fab′-1D4 (inset). Arrowheads indicate single-molecule fluorescence spots. c Kymographic presentation of dynamic heterogeneity of rhodopsin in a disc membrane. Left: pseudocolour image of a disc membrane incubated with -20 nM fluorescent Fab′-1D4. The first frame of a movie is shown. Right: a kymograph obtained by three-dimensional-projection of a 1-s movie on ImageJ. Spatial and temporal scale bars: 2 μm and 0.1 s, respectively. d Tracking of a solitary cluster of rhodopsin. Left: a pseudocoloured snapshot of a disc membrane incubated with 9 nM fluorescent Fab′-1D4. Scale bar: 2 μm. Right: montage of consecutive images (#1–18) from movie of the square area of disc membrane (1 μm × 1 μm) in the left panel. Arrowheads indicate a transient cluster. Frame rate: 30 s⁻¹. e Mean-square-displacement (MSD) vs. time interval (Δt) plot of rhodopsin clusters and estimated diffusion coefficients $D$ (median ± SE; $N = 18$). f Histogram of cluster lifetimes. Lifetime ($τ^*$) was obtained by exponential fitting.
and a non-raftophilic (raftophobic) periphery. To test whether the disc periphery is raftophobic, we examined the distribution of a representative raftophobic phospholipid in the disc membrane, i.e. di-C22:6n3-phosphatidylethanolamine \(^4\): di-DHA-PE (Supplementary Figs. 1 and 5), and compared it with the distribution of rhodopsin (Fig. 6a, b). Unlike the central confinement of rhodopsin, the raftophobic phospholipid exhibited a vigorously fluctuating annular distribution at the edge of the disc membrane (Fig. 6a – c and Supplementary Movie 3). Furthermore, the single-molecule trajectories showed that PE molecules that jumped from...
the rim to the disc central area always returned to the rim quickly (Fig. 6d and Supplementary Movie 4), suggesting that the disc central area has the potential for deterring raftophobic PE.

In accordance with the central confinement of rhodopsin, our semi-multimolecule fluorescence imaging of G\(_{\alpha}\) revealed that G\(_{\alpha}\) is also biased to the disc centre in darkness (Figs. 5a–d, 6e, g and Supplementary Movie 5). In contrast, most of the effector enzyme PDE6 formed immobile hot spots at the disc periphery, and a portion of PDE6 exhibited very fast diffusion (Fig. 6f, g, Supplementary Figs. 1 and 6, and Supplementary Movie 6). This complicated behaviour of PDE6 is consistent with a previous report. Light caused no change in the distribution of PDE6 in the absence of GTP. The bright spot on the upper-left is likely the aggregate of fluorescent G\(_{\alpha}\) at the axoneme. Averaged picture (from 10-s movie) displaying the marginal distribution of PDE6 in disc membrane incubated with ~30 nM fluorescent anti-PDE6 antibody Fab\(^{‘}\)-fragment. g Fluorescence intensity profile along transverse lines in panels (e) and (f). h A montage of averaged pictures indicating lateral translocation of G\(_{\alpha}\) at the disc center along transverse lines in panels (c) and (d).

Discussion
In Supplementary Fig. 7, we present a schematic drawing of the conceptually novel dynamic states of the phototransduction system within the disc membrane. Rhodopsin exists in dynamic equilibrium between three diffusive states presumably ascribable to the cluster raft, dimer and monomer. The slowest diffusive state, accounting for ~20% of rhodopsin in the dark, can be assigned to the rhodopsin-cluster raft. Assuming that the rhodopsin-cluster and the dimer are diffusing in a homogenous lipid membrane (membrane and aqueous viscosities of 8 and 0.2 Poise\(^{22}\), respectively) and that diffusive state-2 is the dimer of 2-nm radius, we estimated the size of a rhodopsin-cluster to be ~90 nm in radius, by using an extended-version Saffman–Delbrück equation (Supplementary Fig. 8). Nevertheless, the large diffusion coefficient of state-3 cannot be explained by the size reduction upon de-dimerization (Supplementary Fig. 8). We may need to assume that the disc membrane has lipid-rich areas differentiated from rhodopsin-dense domains as observed with cryo-electron tomography. Although it has been shown that rhodopsin can form dimers or oligomers with a lifetime estimated to be on the order of 10 \(\mu s\) via protein–protein interactions, our data revealed that the lifetime of a rhodopsin-cluster is on the order of 100 ms (Figs. 1g and 3f and Supplementary Fig. 3a). Considering our results in this paper and previous findings, the high concentration of rhodopsin that tends to self-organize, the dimerization-dependent raftophilicity of rhodopsin, and the coexistence of raftophilic and extremely raftophobic phospholipids, etc. highly likely confer appropriate stability to rhodopsin-clusters.

In addition, it is intriguing that our vbSPT results indicate that the size of the rhodopsin-cluster is kept constant. We conceive
that a delicate balance between protein–protein interactions and the raftophilicity-based stabilizing/de-stabilizing effect of surrounding lipids determines both the lifetime and the size of rhodopsin-clusters. Although molecular interactions that determine the growth, size and stability of receptor-cluster rafts are not fully understood, several key factors are thought to be involved.

Receptor proteins having raftophilic moiety (e.g. GPI-AR) show oligomerization-induced raftophilicity and form receptor-cluster rafts. The receptor clusters that attain raftophilicity can assemble raftophilic lipids, i.e. cholesterol and lipids with saturated alkyl chains. The long, saturated alkyl chains that are in contact with cholesterol tend to be extended through trans-Gauche isomerization of each methylene segment. This alkyl-chain-stretching creates a hydrophobic length-mismatch, relative to the surrounding bulk lipids, driving segregation of receptor-cluster rafts out of the unsaturated bulk lipid membrane phase. Coupled with the tendency for cholesterol to be segregated away from the bulk domain, the line tension of the boundary promotes the assembly of receptor-clusters and increases their size. Suppression of the cholesterol activity of lipids sandwiched within the cluster could also be responsible for stabilizing the cluster. Conversely, the line tension at the interface between the domains of saturated and unsaturated lipids can be reduced by hybrid lipids (so-called linactant) or 2D-detergent, where one chain is saturated and the other unsaturated. This can allow finite-size domains to be stable even in equilibrium. These situations should coincide with that of the rhodopsin-cluster raft. However, the hybrid lipids in the disc membrane account for ~65% of total lipids and having polyunsaturated fatty acid like DHA at the β-position. Therefore, they may destabilize the rhodopsin-cluster rafts by their solubilizing ability. In sum, the lifetime and the size of rhodopsin-cluster is likely determined by a delicate balance of these reciprocal factors, although the exact mechanism remains to be determined. In connection with this, we have previously shown that the dimerization-dependent raftophilicity of rhodopsin essentially requires palmitoyl modifications of rhodopsin. Thus, we had intended to examine the role of palmitoyls in rhodopsin diffusion and distribution. However, in this study, we could not address these issues, because the reducing agents, which break up palmitoyl-cysteine thioester linkages, caused deleterious effects on the shape of f-ROSs.

Given our result that the rhodopsin-clusters in dark-adapted discs tolerate 20 mM MCD, the raftphilic lipids that mainly contribute to the construction of rhodopsin-cluster rafts may not be cholesterol, but, rather, raftphilic saturated-phospholipids such as di-saturated phosphatidylcholine, the main raftphilic phospholipid in the disc. Alternatively, the tolerance of 20 mM MCD may be ascribable to a particular state of cholesterol in the rhodopsin-cluster raft, whereby cholesterol is shielded from the MCD. In fact, about 20% of the cholesterol remains in the disc membrane after 20 mM MCD treatment (Supplementary Fig. 9). Collapse of f-ROSs with higher concentrations of MCD hampered our exploration into the essentiality of the presumably small amount of cholesterol in rhodopsin clustering.

The optimal HMM of rhodopsin in the darkness, in which the medium diffusive state (S2)-dominant, suggests that only a limited amount of rhodopsin forms clusters and that the many remaining rhodopsin molecules are in the dimeric or monomeric state. Such a distribution may be due to the lipid composition of the disc membrane. The raftphilic phospholipid component in the disc membrane is di-saturated phospholipid (mainly 16:0–16:0 phosphatidylcholine), accounting for only 8% of total phospholipid. Thus, the deficiency of raftphilic phospholipids may prevent the incorporation of all rhodopsin into clusters. In this context, diffusive state-2, accounting for 50% of rhodopsin, must be ascribable to metastable rhodopsin dimers that have not been stabilized by such raftphilic lipids, instead, probably being accommodated in the hybrid lipids that account for ~65% of total phospholipid in the disc membrane.

Finally, state-3 might be monomeric rhodopsin, because an appreciable amount of rhodopsin is estimated to exist as monomers, and we observed an extremely large diffusion coefficient, as in state-3, for rhodopsin incorporated in the supported-planar bilayer (Supplementary Fig. 10).

Meanwhile, the high diffusivity of Gt- or IgG-bound rhodopsin in state-3 of the optimal HMM is puzzling. However, it should be noted that IgG-crosslinking does not mean complete fixation of two rhodopsin molecules. Instead, IgG would contribute to stabilizing the rhodopsin dimer by reducing the free volume in the membrane of two rhodopsins via binding to the tips of the C-terminal peptides extended ~5 nm into the cytoplasm. If that is the case, the lipid environment that can destabilize the rhodopsin dimer would cancel the raftphilicity of IgG-crosslinked rhodopsin. Thus, the highly diffusive state seen in HMM of IgG-crosslinked rhodopsin suggests the presence of a region in which rhodopsin dimerization is hindered in the disc membrane. The low-density regions in the disc membrane observed with cryoelectron tomography may coincide with this region. It is also indisputable that the lipid environment may affect the Gt-stabilized rhodopsin-dimerization.

What is the physiological significance of the dynamic non-uniformity of the disc membrane brought about by generation and extinction of rhodopsin-cluster rafts? Taken together, our results support a hypothesis that receptor-cluster rafts likely contribute to achieving the high efficacy of phototransduction. Logically, transient rhodopsin-cluster rafts do not contradict the hypothesis that the array of rhodopsin dimers acts as the ‘signalling scaffold’ or “kinetic trap” responsible for single-photon detection. Pre-associated Gt with a high dissociation rate is expected to quickly scan the array to find Rh*. The lifetime of the rhodopsin-cluster raft (on the order of 100 ms) is sufficiently long compared with the time required for Gt activation by Rh* (~4 ms). A simulation study proposed that longer cluster lifetime and larger cluster size aids higher Gt-activation-efficacy in single-photon regimes. Actually, classical electrophysiological studies have shown that the efficiency of photon capture and conversion into an electrical signal by rods increases with decreasing temperature, by which rhodopsin-cluster rafts could be stabilized. Consistent with this, we found that the diffusive state-1 lifetime increases at a lower temperature (Supplementary Fig. 3). In addition, the raftphilicity of supramolecular structures of rhodopsin can contribute to efficient phototransduction by increasing the lifetime of Rh*, by recruiting a Ca2+-dependent inhibitor of rhodopsin kinase, i.e. recoverin, which has a higher efficacy in the raftphilic environment. It is also known that the base of the ROS contains more cholesterol than does the tip, so the base should be useful for forming rhodopsin-cluster rafts. This hypothesis is consistent with a recent finding that increasing the amplitude of single-photon responses decreases by 5–10 times when illumination of the tip of the ROS is compared with that of the base.

Regarding the supramolecular structure of rhodopsin, to which some Gt weakly pre-associates in the dark, a single photon is expected to be able to activate all of the Gt molecules on the supramolecular structure of rhodopsin in a short period of time. Therefore, the invariance in the size of rhodopsin-cluster suggested in our experiment may be able to explain the constancy in the amplitude of the single-photon response of rod-photoreceptors. This kind of digital-like signalling has also been observed in GPI-AR signalling in cell membrane. In this regard, the constant-sized transient rhodopsin-cluster rafts may
have a role in providing inherently distributed and stochastic platforms that can generate a quite-uniform digital-like response to single photons in rod photoreceptors. This mechanism would also be able to respond to a stronger photic stimulus simply by the summation of digital-like responses.

In addition to its potential importance in phototransduction, the dimerization-dependent raftophilicity of rhodopsin, with resultant raftophilic formation and concentric disc inhomogeneity, may have particular importance in disc morphogenesis and maintenance of rod photoreceptors. It was recently demonstrated that three enigmatic mutants of rhodopsin, known to cause the blinding disease retinitis pigmentosa, inhibit rhodopsin dimerization15. This suggests that, as the nucleating step of rhodopsin self-organization may not occur efficiently in such discs, the supramolecular organization of rhodopsin is compromised with severe consequences for disc architecture and stability.

Our data on the confinement of rhodopsin into the disc central area, and the ring-like distribution of PE in the disc peripheral area implicate the concentric inhomogeneity of the disc membrane in terms of raftophily. We believe that such concentric segregation is based on the hydrophobic mismatch between the hydrophobic length of rhodopsin-cluster raft and the hydrophobic thickness of the lipid bilayer lining the disc rim. Each disc is bounded by a rim composed of tetraspanin complex peripherin-2/rd3-Rom110, where the membrane is distorted into an energetically unfavourable high-curvature bend. It has recently shown that the intrinsically disordered cytoplasmic C-terminus of the peripherin-2/rd3 can generate membrane curvature by associating with cone-shaped lipids such as PE having a small polar head group and bulky polysaturated acyl chains61. Thus, it is conceivable that the rim protein complexes provide a thin framework for the lipid bilayer at the edge of the lamellar region of the disc by recruiting raftophilic phospholipids that reduce the hydrophobic thickness of the bilayer, e.g. di-DHA-PE would make a bilayer of ~25 Å in hydrophobic thickness62. If this is the case, rhodopsin having ~27 Å63 in hydrophobic length and the rhodopsin-cluster, which would have a longer hydrophobic length than rhodopsin, would be excluded from the disc periphery. In support of our hypothesis, AFM shows a protein-free rhodopsin-cluster, which would have a longer hydrophobic thickness of the lipid bilayer lining the disc rim13.

In summary, our single and semi-multimolecule results show that rhodopsin autonomously forms a sort of receptor-cluster rafts, which are in dynamic equilibrium with the lower oligomeric-states of rhodopsin in the disc membrane. Stochastic rhodopsin-cluster rafts provide meso-sized raftophilic signalling platform that is highly likely responsible for the single-photon detection in rod-photoreceptors. Further, the coexistence of multimodal forms of rhodopsin in dynamic equilibrium may allow receptor signalling to have a wide dynamic range, flexibility, and homeostasis of phototransduction machinery. In addition, the concentric heterogeneity in raftophily in the disc membrane may play important roles not only in the regulation of phototransduction but also in maintaining homeostasis of photoreceptor. Together with previously accumulated evidence, our results imply that both the constitutive and the stimulation-dependent clustering of rhodopsin-like GPCRs may have important roles not only in signalling, but also in the biogenesis and maintenance of membrane architecture in the cell, based on their receptor-cluster-raft organizing ability.

Materials. Monoclonal antibody (1D4) against the carboxyl terminus 9-mer peptide of rhodopsin64 was purchased from the University of British Columbia (Vancouver, Canada) via Flimak. Antibody against PDE6a was prepared by immunizing rabbits with a peptide corresponding to the apical end of the PDE6a of Rana pipiens, i.e. 152Asp-154Val: accession number AA953999 (See Supplementary Fig. 1). HiLyte Fluor 750-C2maleimide (HL750-maleimide) and HL750-N-hydroxysuccinimide ester (HL750-NHS) were purchased from Ana Spec Inc. (Fremont, CA). Monoclonal antibody to mouse IgG (κ-light chain) was purchased from Yamasa Corporation (Chiba, Japan). Urea-treated rod outer segment (ROS) membranes, Gt and Gtβγ were prepared from frog ROS membranes as described previously65. di-DHA-PE (1,2-diocosahexaenoyl-sn-glycero-3-phosphatidylethanolamine (PE)) was synthesized by Synpep (Dublin, Ireland) and dilysophosphatidylcholine (DPPC) (1,2-dioleoyl-sn-glycero-3-phosphocholine), DPPC (1,2-di-O-palmitoyl-sn-glycero-3-phosphocholine), chol (cholesterol (ovine wool)), GM1 Ganglioside (bovine brain) and Rh-PE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-lissamine rhodamine B) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholera toxin B-Alexa Fluor 488 conjugate (CTB488) was purchased from Molecular Probes (Eugene, OR). Bullets (Rana catesbeiana) were purchased from Mr. Kazuo Ohuchi (Saitama, Japan). Other standard chemicals were mainly purchased from Merck (Kenilworth, NJ), Fuji Film Wako Pure Chemical (Osaka, Japan), and Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Protease-inhibiting peptides were purchased from Peptide Institute Inc. (Osaka, Japan). Precast sodium dodecyl sulfate (SDS)-polyacrylamide gels (5–20%) were purchased from DRC Co. Ltd (Tokyo, Japan).

Buffers. Standard buffers contained (in mM, unless stated otherwise): buffer A—KCl 60, NaCl 30, MgCl2 3, 3-morpholinopropanesulfonic acid 10, phenylmethylsulfonyl fluoride 0.2, aprotinin 5 ng ml−1, EDTA 10 µg ml−1 (pH 7.5 at 0 °C); buffer B—Tris-HCl (pH 7.5) 10, 1,4-dithio-dL-dithreitol (DTT) 5, MgSO4 6, EDTA 1, MCD 5, and 25% glycerol; buffer C (Ringer’s solution)—NaCl 110, KCl 2.7, MgCl2 2, CaCl2 1, glucose 2, 2-(hydroxyethyl)-1-piperazine-N,N′-bis(2-ethanesulfonic acid) (HEPES)-HCl 10 (pH 7.5); buffer D—k-gluconate 115, KCl 2.5, MgCl2 2.5, BAPTA 0.1, CaCl2 0.01, HEPES-KOH (pH 7.45) 10, taurine 0.2, aprotinin 2 µg ml−1, EDTA 5 µg ml−1 and leupeptin 0.005.

Preparation of frog rod outer segment (ROS) membranes. ROS membranes of bullfrog (Rana catesbeiana) were prepared as described previously29 and stored at 80 °C in the darkness.

Fluorescent labelling of Fab ‘1D4 against the C-terminus of rhodopsin and Fab’ of an antibody against photoreceptor. Antibodies for rhodopsin and 1D4 were illustrated in Supplementary Fig. 1a. The Fab’ fragments were labelled with HL750-C2maleimide as described previously66 and purified by Superose 12 (3.2 × 300) column chromatography on a SMAT system (Pharmacia; Uppsala, Sweden). The dyeprotein ratio was ~ 1.

Fluorescent labelling of Gtβγ. The Ga, was directly labelled with HiLyte Fluor 750-C2 maleimide. The ROS membrane containing 300 nano-mole (~10 mg) of rhodopsin was exposed to room light at 0 °C for 20 min and spun down by ultracentrifugation (100,000 × g, 5 min). Membranes were resuspended with 30 ml of buffer B containing 1 mM MCD. Containing 1 mM MCD (MCD) and 5 mM DTT, and pelleted by ultracentrifugation (170,000 × g, 15 min). This washing procedure was repeated three times. During this procedure, peripheral membrane proteins including rhodopsin kinase (GRK1) were removed67. Then, membranes were suspended and centrifugally washed three times with 30 ml of buffer A containing 20 mM MCD and 5 mM DTT, to remove cGMP-phosphodiesterase 6 (PDE6) and excess Gtβγ68. Resulting membranes were washed with 20 ml of buffer A containing 0.2 mM Tris(2-carboxyethyl)phosphine-HCl (TCEP) for three times in the same manner as above. Finally, the membranes were suspended in 5.28 ml of the same buffer at 0 °C, and quickly mixed with 1.19 × 10−4 M of HL750-maleimide in 300 µl buffer A (TCEP/dye ratio was 1.125 in final concentration)68. The reaction proceeded at 0 °C for 40 min, and was stopped with 25 µl of 1 mM 2-mercaptoethanol. Resulting membranes were washed three times with 20 ml of buffer A containing 1 mM DTT. Finally, HL750-Ga was extracted by suspending the membranes in 1.8 ml of buffer A containing 1 mM guanosine-5′-triphosphate (GTP), 1 mM DTT and 1 mM MCD, followed by ultracentrifugation (452,000 × g, 5 min). This extraction procedure was repeated five times. Pooled extract containing HL750-Ga was applied to a Blue-Sepharose column (handmade; 1 ml in column volume) equilibrated with buffer B, and trapped HL750-maleimide by electrophoresis using a 750-nm scattered laser beam for excitation and a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan; C9100-12) equipped with a long-pass filter and a macro lens (see Supplementary Fig. 1b). HL750-Ga was stored at −20 °C in 50% glycerol. The dyeprotein ratio of puriﬁed HL750-Ga was measured by spectrophotometry to be ~0.7 using ε = 30,400 M−1 cm−1 at 280 nm for Ga-ε, and 250,000 M−1 cm−1 for HiLyte Fluor 750.
Determining the labelling site on HL750-Gq. HL750-Ga, was subjected to gel digestion by Lys-C endoproteinase (Roche, Basel, Switzerland) as described. We used PPI-200 (DHC, Tokyo, Japan) and PGI-200 (DHC, Tokyo, Japan) for desalting of HL750-Ga, and NuPAGE Novex 12% gels (Invitrogen,) for peptide separation. A single major peptide (MW~5000) labelled with HL750 was transferred to a PVDF membrane (see Supplementary Fig. 1c), and the N-terminal amino acid sequence of the peptide was analysed by a protein sequencer (Applied Biosystems, Carlsbad, CA) and compared with predicted LysC-digestion segments of Xcnopus Ga (P38407-1) (see Supplementary Fig. 1a).

Evaluating intactness of HL750-Gq, in light- and GTP-dependent activation. Functional intactness of HL750-Ga, was confirmed by its activation-dependent release from a reconstituted system comprising HL750-Ga, Gαβγ, and urea-treated ROS membrane. Urea-treated ROS membrane containing 50 µg of rhodopsin was incubated with 15 pmol of HL750-Ga, and an equal amount of Gαqβγ, in 50 µl of buffer A containing 1 mM ATP at 0 °C overnight. The ROS membranes were exposed to light for 10 min or kept in the darkness, in the presence or absence of 500 µM GTP and 500 µM GDP. Then the membranes were spun down by ultra-centrifugation at 350,000 x g for 5 min. Proteins in aliquots (8 µl) of supernatants were separated in SDS-polyacrylamide gel electrophoresis, and protein bands containing HL750-Ga, were detected by a hand-made near-IR imaging apparatus (see Supplementary Fig. Id).

Fluorescent labelling of di-DHA-PE. di-DHA-PE was labelled with HL750 SE. 0.6 µM of di-DHA-PE in 50 µl of chloroform was mixed with 2 µl of triethylamine, and then 500 nM of HL750-NHS, dissolved in 5 µl of dimethylsulfoxide, was added. After incubation at room temperature for 2 h, the reaction product was dried by evaporation and dissolved with chloroform:methanolwater (65:25:4). Fluorescently labelled di-DHA-PE was purified on a high-performance thin layer chromatography plate (Merck Millipore, Burlingame, MA, #105641) by developing it with chloroform:methanol:NaOH (65:35:8). The blue band on HPTLC was scraped off from the plate, and the PE was extracted from the silica gel by washing three times with 1 ml of chloroform:methanolwater (65:25:4). The extract was lyophilized to dryness, and dissolved with 1.5 ml of chloroform/methanol (2:1). About 48 µM of HL750-di-DHA-PE was obtained, and kept under N2 atmosphere at ~30 °C.

Preparation of fragmented ROS. All procedures were performed in complete darkness using IR goggles from NEC (Tokyo, Japan). Intact ROS was prepared from the retinas of dark-adapted bullfrogs by the method described previously. Briefly, each retina was separated with a scalpel and epithelium was then placed on three-layered filter papers with the pigment epithelium-side upward. After the vitreous body was absorbed by the filter papers, retinas were cut out using scissors, with a back-up sheet, and kept in buffer C. The retinas with filter papers were placed on a paraffin block covered with Parafilm, attached with several pins, and immersed in 0.8 µM of buffer C per retina. ROSs were detached from the retinal surface by agitating with repetitive pipetting of 50-µl aliquots of buffer C through a large-bore pipette tip (Cell Saver Tip PT-003, InaOptica, Osaka, Japan). Crude ROS suspension was overlaid on a step-gradient of Percoll in buffer C, consisting of 0.6 ml of 70%, 0.3 ml of 50%, and 0.6 ml of 26% Percoll in buffer C, and centrifuged at 17,000 × g for 30 min. The ROS membranes were recovered at the interface between the 44 and 40% Percoll layers. The ROS suspension was diluted with two volumes of buffer D and kept at 0 °C in a light-tight container until use. When we added fluorescently labelled proteins on the disc membrane, we added the proteins in buffer D containing 1 mM 1-ovalbumin to the suspension of 400 µl of f-ROS suspension in buffer D of 0.2–30 nM in final concentration. After 1 h of incubation, the sample was overlaid on a Percoll density-gradient consisting of 0.3 ml of 44%, 0.3 ml of 40% and 0.9 ml of 26% Percoll in buffer D, and then centrifuged at 34,000 × g for 5 min at 4 °C. The f-ROSs were harvested from the interface between the 44 and 40% Percoll layers. The f-ROS suspension was diluted with two volumes of buffer D and kept at 0 °C in a light-tight container until use. When we added HL750-di-DHA-PE to f-ROSs, 10 µl of labelled-PE was evaporated by N₂ gas flow and solubilized with 10 µl of methanol. The methanol solution was diluted with 1 ml of buffer D, and a 5-µl aliquot was added to 500 µl of f-ROS suspension containing approximately 3 nM of rhodopsin. After about 3 h of incubation in ice on the dark, samples were used for experiments. To observe the behaviour of IgG-crosslinked rhodopsin in the disc membrane, we employed FL750–Fab’1D4 antibody, which was purified by Protein A-Sepharose (GE Healthcare, Amersham, UK) and affinity-purified by anti-mouse IgG antibody. The IgG-crosslinked HL750–Fab’1D4 was purified by Superose 12-column chromatography and applied to the f-ROS suspension (~20 nM in final concentration).

Single-molecule imaging. A suspension of f-ROSs with fluorescent probe was introduced into a small chamber made of Secure-Seal from GRACE Bio-Labs (Bend, OR) on a glass slide (Matsunami, Tokyo, Japan) placed in an Attotfluor Cell Chamber from Invitrogen (Paisley, UK). After 10 min, sedimentation of f-ROSs onto the glass surface was complete. The chamber was set on the stage of an inverted total internal reflection fluorescence microscope (TIRFM; Nikon, Tokyo, Japan, TE2000), and the aqueous phase was continuously perfused (0.1 ml min~1) with a buffer containing an oxygen-scavenging system freshly prepared by mixing substrate (2.25 mg ml~1 glucose) and enzymes (216 µg ml~1 glucose oxidase and 36 µg ml~1 catase). The chamber was perfused with freshly prepared perfusion buffer containing 500 µM of GTP. Fluorescently labelled Fab’1D4 on disc membranes was illuminated with the highly inclined laser beam of 750-nm wavelength. Images were acquired with an electron-multiplying CCD camera C9100-12 (Hamamatsu Photonics, Hamamatsu, Japan) at a spatiotemporal resolution of 30 frames s~1 and 78 nm pixel~1. The TIRFM was equipped with Nikon 100×/1.45 Plan-Apo objectives. A filter setting consisting of 760DRPL (Chroma Technology Corp., Bellows Falls, VT) and HQ810/90 (Omega Optical) was used.

Single-molecule tracking. Coordinate points of fluorescent spots were measured with TrackMate on Fiji (http://fiji.sc/TrackMate) using a Laplacian of Gaussian detector for segmentation on the image and a Simple Linear Assignment Problem (LAP) tracker for the particle-tracking algorithm. Localization error was assessed to be ~50 nm by single-molecule tracking of immobile spots of fluorescently labelled proteins on a glass surface. Splitting and merging events were ignored. Effective diffusion coefficients of single fluorescent molecules within 100 nM (D_ros) were evaluated by mean square displacement (MSD)-time intervals (A(t)) using a per- user prepared Matlab toolbox from Tinevez’ lab (http://tinevez.github.io/msdanalyzer/). Whole trajectories (minimum trajectory length >15 frames; average trajectory length ~35; average number of trajectories ~400) obtained by TrackMate were imported into msdanalyzer. We eliminated trajectories yielding bent curves, which likely resulted from partial confinement of membrane molecules by incisions of the disc membrane, based on the criterion of good-enough-fitting (R^2 > 0.8) of the MSD–Δt curve (http://tinevez.github.io/msdanalyzer/tutorial/MSDValidationtutorial.html).

Variational Bayes single-particle tracking (vbSPT) analysis of single-molecule trajectories. Time-series data provided by single-molecule experiments offer the opportunity to infer not only model parameters describing transition rates between molecular states, but also information about the model itself, e.g., the number of molecular states. If the complex of interest transitions from one locally stable diffusive state to another, the experiment is well-modelled by a hidden Markov model (HMM)6,72, a probabilistic model in which an observed time series is conditionally dependent on a hidden, or unobserved, discrete state variable. To extract effective states and transition rates of rhodopsin diffusing in retinal disc membranes, we applied an open-source software vbSPT (http://www.sourceforge.net/projects/vbsp/) to single-molecule tracking data used in MSD–Δt analysis. The number of iterations and bootstraps were set to 25 and 100, respectively. The vbSPT method uses a maximum-evidence criterion to determine the underlying number of molecular states and the number of diffusive states from the observed data. We validated vbSPT using simulated reaction diffusion trajectories in a disc geometry (a circle 8 µm in diameter) and in a 1 x 2 µm area mimicking a small lobule of frog disc membrane, with the same trajectory length distribution as that of our experimental data and using realistic localization errors (50 nm), diffusion coefficients and transition parameters (Supplementary Table 2). The method successfully recovered the parameters used for simulating the data.

Semi-multiparticle fluorescent imaging of rhodopsin, Gαs, PDE6 and PE on disc membranes. To observe the collective behaviour of rhodopsin, Ga, and PDE6 in disc membranes, we used f-ROSs incubated with approximately 10–30 nM of HL750-Fab’1D4, HL750-labelled Ga, or HL750-Fab’ of anti-PDE6 antibody. By using approximately 100 mW of 750 nm wavelength, there were no differences between the semi-multiparticle fluorescent imaging and single-molecule imaging. To observe the collective behaviour of a highly raftophobic cone-shaped phospholipid, PE, we used HL750–di-DHA-PE in chloroform:methanol:NaOH (65:35:8). The disc material was dissolved in methanol at 60 °C and dispersed into the f-ROS suspension, to be 4.8 nM in final concentration. Following 30 min incubation, we performed semi-multiparticle fluorescent imaging of di-DHA-PE in the disc membranes.

Determination of D_ros of rhodopsin clusters. Coordinate points of fluorescent spots were measured with TrackMate on Fiji using a Laplacian of Gaussian detector for segmentation on the image and a Simple LAP tracker for the particle-tracking algorithm, and manually edited after inspection. D_ros was evaluated by the per-value class “msdanalyzer” on Matlab as described above.

Photosensorization of rhodopsin in discs. The suspension of f-ROSs in the perfusion chamber was illuminated by a flashlight (Contax TLA140 from Kyocera, Kyoto, Japan) through a green filter OG535 from Schott AG (Mainz, Germany). The amount of isomerization was determined by the change in the absorbance at 550 nm. The ROS suspension on the chamber on TIRFM was illuminated for a bright light flash. Then, we obtained arbitrary light intensity by using neutral filters.

Manipulation of membrane cholesterol content. To deplete cholesterol in the disc membranes for single-molecule tracking experiments, f-ROSs were suspended in...
in buffer A containing 20 mM MCD. Treated f-ROSs were isolated by Percoll density-gradient centrifugation using 44, 40, and 26% step gradients of Percoll as described above. The cholesterol content was decreased by about 80% by the MCD treatment (see Supplementary Fig. 9), as assessed by Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific, Waltham, MA). To replenish cholesterol, cholesterol-depleted f-ROSs were incubated with buffer A containing 0.6 mM cholesterol and 20 mM MCD for 1 h at 0 °C in complete darkness. Cholesterol-replenished f-ROS was spun down by gentle centrifugation (100 × g for 3 min) and resuspended with buffer A. All procedures were performed in complete darkness.

**Determining the diffusion coefficient of monomeric rhodopsin in a fluid liquid bilayer membrane.** Rhodopsin was reconstituted into a preformed supported planar bilayer (SPB) membrane, composed of 1-palmitoyl-2-oleoyl-phosphatidylcholine, by the rapid dilution of detergent-solubilized Cy5-labeled rhodopsin. Single-molecule tracking was performed on TIRFM, and obtained trajectories were subjected to MSD analysis to determine $D_{omv}$.

**Assessment of the raftophic nature of NL750-di-DHA-PE.** The raftophlicity of fluorescently labelled di-DHA-PE was assessed based on its distribution into liquid-disordered ($L_d$) and liquid-ordered ($L_o$) phases artificially formed on the SPB membrane74. Patterned separation of $L_d$ and $L_o$ phases was induced in DOPC:DPPC:Chol (1:1:1) (with GM1 and Rho-PE (1% each)). Ten microliters of 4.8 nM sample was injected into the SPB membrane, and obtained trajectories were subjected to MSD analysis to determine $D_{omv}$.

**Statistics and reproducibility.** Statistical analysis was performed using MatLab. Differences between multiple groups were assessed by one-way or two-way analysis of variance (ANOVA), as appropriate, followed by Tukey’s multiple-comparison test. Differences between two histograms were assessed with the Mann–Whitney U test.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Data that support the findings of this study are available from the corresponding author upon reasonable request. Raw data used to generate the plots can be found in Supplementary Data 1 file accompanying this manuscript.

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Author contributions

F.H. conceived and coordinated the study. F.H., K.S., and N.S. performed experiments. F. H. performed data analysis and wrote the paper with K.M., K.S., and N.S.Y.T., K.O., and K.M. performed artificial bilayer lipid membrane experiments. S.M. supervised the biochemical studies, as well as the overall project design and execution. All authors reviewed the results and approved the final version of the manuscript.

Additional information

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