Defining raft domains in the plasma membrane

Akihiro Kusumi[1,2], Takahiro K. Fujiwara[2], Taka A. Tsunoyama[1], Rinshi S. Kasai[3], An-An Liu[4], Koichiro M. Hirosawa[5], Masanao Kinoshita[6], Nobuaki Matsumori[6], Naoko Komura[5], Hiromune Ando[5], and Kenichi G. N. Suzuki[5]

[1]Membrane Cooperativity Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Okinawa 904-0495, Japan. [2]Institute for Integrated Cell-Material Sciences (WPI-iCeMS) and [3]Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan. [4]Tianjin Key Laboratory of Biosensing and Molecular Recognition, Research Center for Analytical Sciences, College of Chemistry, Nankai University, Tianjin 300071, P. R. China. [5]Center for Highly Advanced Integration of Nano and Life Sciences (G-CHAIN), Gifu University, Gifu 501-1193, Japan. [6]Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 819-0395, Japan.

Send correspondence to:
Akihiro Kusumi
Okinawa Institute of Science and Technology Graduate University (OIST)
Okinawa 904-0495, Japan.
E-mail: akihiro.kusumi@oist.jp

Abstract

Many plasma membrane (PM) functions depend on the cholesterol concentration in the PM in strikingly nonlinear, cooperative ways: fully functional in the presence of physiological cholesterol levels (35~45 mol%), and nonfunctional below 25 mol% cholesterol; namely, still in the presence of high concentrations of cholesterol. This suggests the involvement of cholesterol-based complexes/domains formed cooperatively. In this review, by examining the results obtained by using fluorescent lipid analogs and avoiding the trap of circular logic, often found in the raft literature, we point out the basic similarities of liquid-ordered (Lo)-phase domains in giant unilamellar vesicles, Lo-phase-like domains formed at lower temperatures in giant plasma membrane vesicles, and detergent-resistant membranes: these domains are formed by cooperative interactions of cholesterol, saturated acyl chains, and unsaturated acyl chains. The literature contains good evidence, showing that the domains formed by the same basic cooperative molecular interactions exist and play essential roles in signal transduction in the PM. Therefore, as a working definition, we propose that raft domains in the PM are liquid-like molecular complexes/domains formed by cooperative interactions of cholesterol with saturated acyl chains as well as unsaturated acyl chains, due to saturated acyl chains' weak multiple accommodating interactions with cholesterol and cholesterol's low miscibility with unsaturated acyl chains and TM proteins. Molecules move within raft domains and exchange with those in the bulk PM. We provide a logically established collection of fluorescent lipid probes that preferentially partition into raft and non-raft domains, as defined here, in the PM.

1. Introduction

The hypothesis of lipid rafts in the membrane was first proposed in a review by Kai Simons and Gerrit van Meer in 1988, in the context to explain the distinct trafficking of specific proteins from the Golgi to the apical plasma membrane (PM) in polarized simple epithelial cells.[1] Although they did not use the word "raft", the key raft concept was presented. They proposed that glycosphingolipids and sphingomyelins are clustered to form a microdomain in the luminal leaflet (which could become the exoplasmic leaflet in the PM) of the bilayer of the trans Golgi membrane, and this microdomain functions as the platform to concentrate apical proteins (by unknown mechanisms) and the budding site to produce apical membrane vesicles.

The lipid raft concept was clearly proposed in a review by Simons and Ikonen published in 1997:[2] the rafts are dynamic clusters of sphingolipids and cholesterol, which move within the fluid bilayer and function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction. Since then, the lipid raft concept has been constantly revised to become consistent with newly obtained data.[2-13] This journal, *Traffic*, originated in 1999, and so its growth has occurred contemporarily with advancements in lipid raft research. Therefore, it is quite meaningful to include a review on lipid raft domains in the 20th Anniversary Issue of *Traffic*. We feel quite honored to review the current understanding of lipid raft domains, based on the results obtained over these past 20 years or so, and provide future perspectives of the field in this 20th Anniversary issue. Our group published a review about raft domains in 2004 in this journal,[4] and so it is our great pleasure to see the impressive advancements in this research field during the past 15 years.

Our previous reviews,[4,10-12] particularly the one published in this journal in 2004,[4] contain important descriptions of the cholesterol interactions with saturated and unsaturated acyl chains and transmembrane (TM) proteins, as well as how the actin-based membrane skeleton mesh (the part of the cortical actin filaments bound to the PM cytoplasmic surface) interacts with (presumable) raft domains. The knowledge of these interactions is quite essential for understanding this review and the raft domains. However, in this review, we simply state these key interactions without describing their supporting data, because we hope to avoid being too repetitive and these interactions are now quite well known.

The definition of the lipid raft domain is still being developed, but the current general

view of the raft domains in the PM (not the definition) could be summarized as follows (most of the references for these statements will be given later).

"The raft domains in the PMs of *quiescent cells* are meso-scale domains of 2 - 20 nm in diameter, in which cholesterol and molecules containing long saturated acyl chains are assembled in the liquid-ordered [L_o]-phase-like organization, due to the cooperative interactions between cholesterol and long saturated acyl chains and their exclusion from (their immiscibility with) unsaturated acyl chains, in a mechanism likened to phase separation. This way, raft domains concentrate the molecules exhibiting better miscibility with L_o-phase-like domains (recently, Lorent et al.[14] quantitatively clarified the factors determining the miscibility of TM proteins in raft domains). The raft domains maintain liquid-like properties, allowing the assembled molecules to diffuse within the raft domain and exchange with molecules in the bulk domain, which is in the liquid-disordered [L_d]-phase-like state. The residency lifetimes of individual molecules recruited to the meso-scale raft domain are often on the order of fractions of a second (The lifetimes of the metastable raft domains themselves have not been clarified)."

"*Upon stimulation* of raft-associated receptors (or proteins), the engaged receptors are clustered, with the aid of the raft domains pre-existing in the quiescent cells, thus inducing the coalescence and stabilization of the raft domains. These stabilized raft domains function as the platforms for signal transduction, interactions with cortical actin filaments, endocytosis, and other phenomena.[4,9,11,12,15] The ligated receptors might be clustered for longer durations, but the lipid components of the stabilized raft domains and the lipid-anchored signaling molecules recruited there (are likely to maintain high mobilities and) undergo rapid exchanges with those in the bulk domain, with residency lifetimes on the order of fractions of a second."

As indicated in these statements, we emphasize the importance of separately considering the transient (metastable) raft domains in the steady state and the activated/engaged raft domains after stimulation. The raft domains "isolated" from animal tissues by detergents are likely to be the stabilized, activated raft domains, because the raft-associated receptors in the cells in tissues are considered to constantly receive signals from other cells and hence are often ligated and engaged. This concept of the meso-scale (generally 3 – 300 nm in size) functional subcompartments in the PM was powerful and intuitively appealing, and thus it has now infiltrated virtually all areas of cell biology.[16] Caveolae could be

considered as an important special example of such stabilized raft domains.[17] For caveolae organization and function, see the splendid reviews by Parton.[18,19]

However, note that this summary statement about raft domains does not provide the "definitions" of lipid raft domains. Consider the following questions. What kind of experiments can we do, if we suspect that a biological function we are interested in might be enabled by the involvement of raft domains and wish to prove it? What advice could we give to our colleague who suspects that the PM substructure s/he is interested in, for example, desmosomes, adherens junctions, and synapses, as well as their subdomains, might involve raft-lipid interactions and wants to test this working hypothesis? To address these questions, the explanations of raft properties described in the previous paragraphs would not be directly useful. We would need a (working) definition of lipid raft domains at high levels of accuracy and logical correctness, as well as testability by relatively simple experiments.

With the development of liquid-liquid phase-separated giant unilamellar vesicles (GUVs),[20-26] giant PM vesicles (GPMVs and PM spheres),[27-29] new fluorescence lipid analogs,[30,31] and single-molecule imaging and superresolution fluorescence microscopy,[13,32-40] as well as the clarification of the key structural parameters of TM proteins for their inclusions in raft domains,[14] we believe that the time is now ripe to synthesize our current biological, chemical, and physical knowledge of raft domains. This knowledge should be reorganized in a logical way (the problem of the circular logic in the raft research field is explained in the next section, Section 2) and provide a simple working definition of raft domains that will be practically useful for further studies. This review is an attempt to accomplish these goals. The end results of this attempt might not be very different from what we think we know overall, which would indeed be a good thing for the field. However, having a simple clear working definition of raft domains in the PM ("definition" and not the explanation of the properties of raft domains), such as that we propose here, would be important for further development of the field. Furthermore, we tried to comprehensively reorganize the existing data obtained by fluorescent lipid analogs, which we think are key tools for studying raft domains in the PM, and point to which analogs should be used to directly find raft domains in the PM in living cells at physiological temperatures. We hope this will be useful for specialists as well as beginners in this research area, and for research in the broader areas of cell biology, biophysics, and biochemistry.

In this review, we will focus on the raft domains located in the PMs of mammalian cells, because these domains are the best studied. However, we believe that the organized knowledge of such raft domains could also be used as a basis for understanding other raft domains, such as those in the Golgi or in other animal and plant species (for reviews of lipid-domain-dependent protein sorting and transport, see Refs. 41 and 42).

2. Why has it been difficult to define raft domains? Technological and logical problems

We think there are two reasons why it has been difficult to define raft domains in the PM. The first reason is that raft domains in live cells, particularly those in quiescent cells, have never been visualized or isolated in ways that the research community can agree upon. Then, why has it been difficult to visualize rafts in quiescent cells? Looking back on the 30-year history of raft research, the reason is rather clear now. The raft domains are small, with sizes mostly less than 20 nm in quiescent cells and often less than 200 nm even after stimulation. Such small sizes make visualization in living cells difficult, even when using the most advanced super-resolution fluorescence microscopy methods. Furthermore, the small size is linked to the fact that the number of the same protein species located in a single raft domain might often be 0 or 1. Even if 5 copies of a protein species existed in a single domain, dual-color super-resolution and immunoelectron microscopy methods would rarely detect the colocalization at levels with statistical significance, and thus would not be able to detect domains or molecular complexes. Some lipid species might be more abundant in raft domains, but the same raftophilic lipids are also likely to exist in the non-raft domain at quite high concentrations at the physiological temperature of 37°C. Coupled with the small sizes of raft domains (<20 nm in quiescent cells) and the existence of very large amounts of endogenous natural lipids, identifying raft domains by fluorescent lipid analogs would be difficult. In addition, proper fluorescent lipid probes that behave like the endogenous parental lipids that form raft domains in the PM had hardly been available until quite recently.[30,31] The dwell times of raft-associated molecules in a raft domain might be on the order of milliseconds to several 10s of milliseconds,[30,31,43-45] which make the direct observations of raft domains quite difficult. The method of isolating raft domains, by

taking advantage of detergent-resistant membranes (DRMs), has remained to be reevaluated for its validity.[3,15,46-49]

The second reason is the problem of circular logic prevalent in the raft research field. This problem was probably induced because the field was initiated by a powerful raft hypothesis, which, when coupled with a simple biochemical procedure to prepare DRMs,[50-52] quickly penetrated virtually all aspects of molecular cell biology. Researchers indeed found a variety of microdomains with important functions, which could be loosely linked to DRMs and the clusters of sphingolipids and cholesterol originally proposed by Simons and Ikonen.[2] These were splendid advancements for membrane molecular biology and biochemistry, but it necessitated new and expanded definition(s) of raft domains (although some doubts about the equality of raft domains and DRMs have been expressed[3,15,46-49]). This way, the field of raft research initiated a voyage to broaden its horizon and at the same time to define the horizon. Namely, the research field of lipid raft domains in the PM has become the field where the researchers seek the correct as well as useful definition of raft domains.

Partly due to the lack of a simple definition of raft domains in the PM, and partly due to the technical difficulties, the prevalent logic in the raft-domain research field has often been that "we found new raft domains in the PM, using a probe molecule that is believed to partition into raft domains (meaning that, based on the general feel of the field, the probe is "considered" to partition into some raft-related domains, although without any direct evidence)". This is a logical fallacy, because one must independently prove that the probe actually preferentially partitions into the raft domain (or non-raft domain). However, what we want to assert here is that even with the problem of logical circularity, the logic circles were generally quite large in this research field, and thus useful knowledge has been accumulated over these 20~30 years for discovering the true nature of this interesting PM domain. Therefore, for the healthy development of the raft-domain research field in the future, it is now important to re-examine the literature and reconstruct/establish the straightforward logic in the raft-domain research.

For this purpose, we start this review by explaining that many signaling processes in the PM and other PM functions depend on the cholesterol concentration in strikingly nonlinear, cooperative ways. They are fully functional in the presence of 35~45 mol% cholesterol (against the total lipid) in the PM, but totally lose their functions when the cholesterol concentration is

reduced to ~25 mol% or less, i.e., still in the presence of high concentrations of cholesterol in the PM. This suggests the existence of lipid complexes/domains that are formed cooperatively with respect to the cholesterol concentrations in the membrane. Therefore, we look for such lipid complexes/domains in GUVs, GPMVs (PM spheres), and DRMs (and in some cases, in the antibody-induced GPI-anchored-protein domains in the PM). We organize the data in the literature, and point out the basic similarities of the Lo-phase domains in GUVs, Lo-phase-like domains in GPMVs, and DRMs, and ask whether the domains formed by the same basic cooperative interactions of cholesterol and acyl chains, found in GUVs, GPMVs, and DRMs, actually exist in the PM. If such domains exist, then we could define such domains as lipid raft domains in the PM (as a working definition). We next identify the properties of such domains and their functions in the PM.

For this objective, and also for the practical purpose of actually performing experiments in future research, we extensively examined fluorescent lipid analogs. This is also a reasonable approach, since using fluorescent lipid probes is one of the key strategies in raft domain research.[25,53-57] First, we first searched for molecules that have been *independently* shown to partition into Lo (Ld)-phase domains in GUVs as well as the Lo (Ld)-phase-like domains in GPMVs, to escape from the circular reasoning loop. Indeed, we found in the literature that lissamine rhodamine B sulfonyl-dioleoylphosphatidylethanolamine (LRB-DOPE) satisfies these conditions (preferring Ld-phase[-like] domains; Table 1).[58] Surprisingly, except for LRB-DOPE, we could not find any other fluorescent lipid analogs that were *independently* shown to preferentially and robustly partition into Ld-phase(-like) domains in both GUVs and GPMVs in the literature. Furthermore, by making quite reasonable assumptions that other dioleoyl and dilinoleoyl phospholipid probes behave like LRB-DOPE in both GUVs and GPMVs, we found that these probes partition into non-DRM. Thus, by starting from LRB-DOPE, we were able to sort and reorganize the partitioning data for other fluorescent lipid analogs, so that we can escape from the situation where the premises are mixed with the conclusions (without falling into the trap of circular arguments). Since this is done here (and once this were done properly), one could pick any warrantied lipid probes from the list shown here and use them as true bona-fide markers for Ld (Lo)-phase(-like) domains in GUVs and GPMVs, and probably for the (non-)raft domains in the PM. Previously, various "bona-fide" markers have been used in the sense that

their partitioning behaviors are consistent with each other in the literature, but this was indeed the very source of the circular logic and sometimes it was the sources of incorrect uses of lipid probes (particularly the lipid probes considered to be raft-associated; see Table 1).

Fluorescently-tagged GPI-anchored proteins are also excellent probes for defining the raft domains, but due to space limitations, we will touch upon their uses only briefly in this review (The functional importance of GPI-anchored receptors has been summarized in a wonderful review of Thy-1 by Morris).[59]

3. Many signaling processes in the PM depend on the cholesterol concentration in strikingly nonlinear, cooperative ways

The cholesterol concentration in the PM of mammalian cells, as expressed as the mol% of total lipid (largely, phospholipids + gangliosides + cholesterol) is generally around 35~45 mol%.[9,60] When cholesterol is partially and mildly depleted by incubating the cells with methyl-β-cyclodextrin or saponin (for example, an incubation with 4 mM methyl-β-cyclodextrin at 37˚C for 30 min), the cholesterol concentration is typically decreased by 40% to become 21~27 mol%.[61-63] For simplicity of presentation, we assume that the overall cholesterol concentration in the PM is 40 mol%, and after mild cholesterol depletion, it is reduced by ~40% to become 25 mol%. Since cholesterol is such an abundant molecular species, one might expect that if a PM function is dependent on cholesterol, then upon cholesterol depletion, the signaling reaction rate at the PM might be reduced by ~40% (reduced to 60% of the original level).

On the contrary, many signaling reactions became almost totally blocked after partial cholesterol depletion. Examples include the formation of the αvβ3-integrin/CD47/trimeric G protein complex and its downstream signaling,[61] the phosphorylation of the engaged FcεRI by Lyn kinase,[64] NK1 receptor activation,[65] P2X1 receptor-mediated currents,[66] tumor necrosis factor receptor 1 signaling,[67] CD59-induced activation of the trimeric G protein, Lyn, and IP_3-Ca^{2+} pathway,[63,68] and dectin-1 signaling,[69] as well as the entry of *Listeria monocytogenes*[70] and anthrax toxin[71].

The nonlinear responses of these reactions with respect to the cholesterol concentration

in the PM clearly indicate that these reactions do not depend on simple interactions with single cholesterol molecules. If these reactions had depended on simple interactions with cholesterol, then the reactions would have been reduced only to 60% (rather than almost to 0%) of the original level when the cholesterol concentration was reduced from 40 mol% to 25 mol%. Rather, these results suggest that the reactions must depend on the domains/structures in the PM that are cooperatively formed in the presence of overall cholesterol concentrations higher than 25 mol% at 37°C. We will have to find such structures or domains in the PM (In the papers cited in this section, the authors used the term "raft". However, this does not affect the logic here. If this is a concern for the readers, please rephrase these words as "presumable raft". Note that we do not use the term "raft" in this section). We will do this in the following part of this review, based on the results obtained by using GUVs, GPMVs (PM spheres), and DRMs.

Partial cholesterol depletion could generate the side effect of enhancing actin polymerization by reorganizing phosphatidylinositol 4,5-bisphosphate (PIP_2),[72,73] but when this occurs, the effect reportedly lasts for at least 12 h (up to 24 h).[72] Therefore, when cholesterol repletion restores the original level of PM functions quickly,[63,65,68,70,74] it suggests that the blocking of the PM function by the partial cholesterol depletion is likely due to the direct effect of lowering the cholesterol level by ~40%.

Liu et al. recently developed an imaging method for quantifying cholesterol in two leaflets of the PM in live cells, and found that cholesterol concentration in the inner leaflet is ~12-fold lower than that in the outer leaflet, whereas the mole fraction of cholesterol in the PM was 22 mol%.[75] This suggests that the cholesterol mole fractions in the outer and inner leaflets will be 41 and 3.4 mol%. Although the result obtained by Liu et al. is quite different from the previous results, showing that cholesterol is more concentrated in the inner leaflet,[76,77] their result indicates that the argument here would be useful in considering raft domains in the PM outer leaflet. Liu et al. further showed that keeping the cholesterol level low in the inner leaflet in quiescent cells is critical for triggering cholesterol-dependent Wnt signaling.[75]

4. Giant unilamellar vesicles (GUVs): The Lo-phase-like domain might

be involved in the nonlinear dependence of raft-related PM functions on the cholesterol concentration

What are the molecular complexes or domains in the PM at 37˚C that depend on the bulk cholesterol concentrations higher than 25 mol% for their existence? To find the answer, let us turn to artificial giant unilamellar vesicles (GUVs) composed of a ternary mixture of cholesterol, a saturated phospholipid, such as L-α-dipalmitoylphosphatidylcholine (DPPC) or L-α-distearoylphosphatidylcholine (DSPC), and sphingomyelin (18:0, SM), and an unsaturated phospholipid, such as L-α-dioleoylphosphatidylcholine (DOPC). Such GUVs would serve as the simplest models of the PM.

Their phase diagrams have been produced by observing the partitioning and dynamics of fluorescent, paramagnetic spin, and deuterated probes.[20-26,78,79] The results indicated that the liquid order-disorder phase separation (liquid ordered and disordered phases are called the Lo phase and Ld phase, respectively, in this review) occurs with quite specific molecular compositions (nearly equimolar ratios of cholesterol and saturated and unsaturated phospholipids) at temperatures at and below the physiological temperature of 37˚C. At 37˚C, the molecular fractions at which the Lo-Ld phase separation could be induced are quite limited in the ternary mixtures of DSPC(DPPC)/DOPC/cholesterol,[23] and the cholesterol concentration must be higher than 25 mol%. The phase diagrams of SM(18:0)/DOPC/cholesterol at 37˚C are not available to the best of our knowledge, but the phase diagrams obtained at several lower temperatures and/or using SM(16:0) suggest that the Lo-Ld phase separation occurs mostly when the cholesterol concentrations are not much lower than ~20 mol%[21,24,78] (see also the wonderful collection and well-considered presentation of the three-component phase diagrams by Marsh[80]).

Fluorescence microscopy revealed that both the Lo- and Ld-phase domains in GUVs are often on the micron-scale (Fig. 1).[21-24] Since the phase diagrams obtained by fluorescence microscopy, which only gives a spatial resolution of ~300 nm, agree well with those obtained by molecular-level techniques such as deuteron NMR and EPR spin labeling,[78,81] the micron-scale domains are likely to represent the Lo- and Ld-phase domains. However, note that these domains are not always on the micron-scale. In particular, when DOPC was replaced by 1-

palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), even at compositions where the fluorescence images appear uniform, nanoscopic domains (≥5 nm in diameter) with behaviors closely resembling those of the genuine phases were detected.[82] In equilibrium thermodynamics, the minimal number of molecules required to form a structure that can be defined as a phase might be around 10, because, although this is a hand-waving argument, the error by using the most approximate Stirling's formula of log[n!] ~ nlog[n] -n becomes less than 15% when n ≥ 10. Furthermore, note that the micron-scale "Lo-phase domain" detected by fluorescence microscopy might not always be uniform: the ultrafast interferometric single-particle tracking (with a 20 μs resolution) of a 20-nm gold particle bound to a phospholipid detected the presence of nano-subdomains as small as 10 nm within the Lo-phase, which transiently trapped the phospholipid.[83]

The two micron-scale domains found in GUVs were found to be liquid domains in three ways. First, fluorescent lipid analogs and a GPI-anchored protein, placental alkaline phosphatase (PLAP) exhibited different diffusion coefficients in two coexisting domains in GUVs.[21,31,84,85], and both were comparable to those found in the Lo- and Ld-phase domains in simpler artificial membranes (summarized in Table 2).[21,84,86] Second, fluorescent C-Laurdan (6-dodecanoyl-2-methylcarboxymethylaminonaphthalene)[87-89] exhibited the presence of two domains with different orders (lipid packing), but both orders were lower than that in the solid-phase membrane[58]. Third, in the observations of two coexisting domains near the miscibility transition temperature, the shapes of the domains were changing continuously.[22,24]

How well ordered is the Lo-phase domain? A deuterium NMR study revealed that the lipid alkyl chain order between C4 and C16 is substantially higher (lower) in the Lo-phase domain than in the Ld (solid)-phase domain.[79,81] The order evaluated by using fluorescent C-Laurdan showed that it is much higher in the Lo-phase domain than in the Ld-phase domain.[53,58]

The phase diagrams demonstrated that the Lo-phase domain largely consists of saturated phospholipids and cholesterol. Based on thermodynamic modeling, Radhakrishnan and McConnell[90] concluded that the Lo-phase domain contains high concentrations of condensed 1:1 complexes of cholesterol and saturated PC, and that this condensed complex is immiscible with unsaturated PC (DOPC). In addition, somewhat stable molecular complexes of saturated PC and cholesterol at molar ratios of 4:1 and 2:1, as well as 1:1, have been

reported,[20,91-93] suggesting that the Lo-phase domain would contain such complexes.

Taken together, the GUV results indicate that the Lo-phase domain containing higher cholesterol mole fractions as compared with the bulk mole fraction could be cooperatively formed even at 37˚C, when the bulk cholesterol concentrations are higher than 25 mol%. This result suggests that "the PM domains resembling the Lo-phase domain, in which saturated acyl chains and cholesterol exist in high concentrations and form molecular complexes, might be responsible for the non-linear dependence of many PM functions on the overall cholesterol concentrations higher than 25 mol%".

The cause for such Lo-Ld phase separation has been proposed to be the weak multiple interactions of cholesterol with saturated acyl chains, due to the conformational conformability of saturated acyl chains (the rotational-conformational freedom of all of the single C-C bonds), as well as the lateral non-conformability (immiscibility) of cholesterol with unsaturated acyl chains.[4,10,94-97] Due to the rigid, planar tetracyclic sterol backbone of cholesterol and the rigid, mandatorily-bent cis-double bonds in the unsaturated acyl chain, at least at the C9=C10 position, placing cholesterol and unsaturated acyl chains laterally next to each other would be energetically less favorable, as compared with the case where cholesterol is placed next to saturated acyl chains. Therefore, if given a choice within the GUVs, cholesterol would be segregated out from the domain composed of unsaturated acyl chains (plus some saturated chains, because saturated chains can mix with unsaturated chains), taking the saturated acyl chains along because cholesterol by itself cannot form a membrane, and thus forming the domain made of cholesterol and saturated acyl chains (note that cholesterol can stay to certain extents in domains largely consisting of unsaturated acyl chains; in unsaturated lipid domains, cholesterol tends to form small clusters of several cholesterol molecules with lifetimes of 1-100 ns[94]). Namely, cholesterol can form (transient) complexes with saturated acyl chains (for example, acyl chains of phospholipids, GPI-anchored proteins, glycosphingolipids, and sphingomyelins), even if the affinities between cholesterol and these molecules are not particularly high (due to the exclusion from the domains largely made of unsaturated chains). This mechanism can explain why cholesterol and saturated acyl chains form a liquid-ordered domain. Since saturated acyl chains interact with cholesterol's rigid, planar tetracyclic sterol backbone, the acyl chains tend to have more trans conformations (thus becoming ordered) and

since cholesterol and acyl chains may not bind strongly, they could move about in their concentrated domains (thus being in liquid states).

5. Determining the Lo- and Ld-phase preferences of fluorescent lipid probes in GUVs

In the examinations of GUVs using fluorescent lipid probes, we can often determine whether and how the employed probe preferentially partition into the Lo/Ld-phase domains from the temperature dependence of the area fraction and connectivity[25,98], diffusion coefficient in each domain,[21,31] and partitioning of fluorophores with known partitioning behavior. The last method, examining colocalized and/or complementary staining using fluorescence microscopy, is quite prevalent, because it is simpler and virtually the same method can be used for the examination of GPMVs. However, to make this method work without falling into the trap of circular logic, we first need to find at least one fluorescent probe that preferentially partition into the Lo- or Ld-phase domain (in fact, also in Lo- or Ld-like domains in GPMVs), without using the data of simple colocalization or complementary distribution with another molecule.

See the first and second (GUVs') columns in Table 1, and the first row there. Kaiser et al.[58] found that, in GUVs, the domains more intensely labeled with LRB-DOPE are colocalized by C-Laurdan's lower polarity region (more disordered domain), which establishes that LRB-DOPE is an Ld-phase preferring probe in GUVs. To the best of our knowledge, such a clear Ld-phase preference by experimental design and results had not existed for any other fluorescent lipid probes in the literature, until recently. Without these observations, it would have been impossible for us to start logical reorganization of the partitioning data in the literature.

Other lipid probes containing two oleoyl chains and those containing two linoleoyl chains are expected to behave similarly to LRB-DOPE and DOPC (which is the most prevalent host molecule for the ternary lipid mixture to produce phase-separated GUVs), due to the presence of one and two unsaturated cis bond(s), respectively, which induces lateral nonconformability with cholesterol. Therefore, these lipid probes were also categorized into Ld-phase preferring molecules (Table 1, first row). Indeed, this is the only speculative part in Table 1.

Starting from these observations, we reorganize the lipid partitioning data, without being

trapped in the circular logic, examining the complementary and colocalized partitioning with respect to the lipid probes with established partitioning behaviors in the logical order (from the top row toward the bottom row in Table 1). See the second row in Table 1. Since the CTB-GM1 pentamer complex exhibited complementary staining with LRB-DOPE, it was categorized as a "Lo-phase preferring" molecule.

Meanwhile, 488neg-SM(18:0) (Hd; indicating the probe attachment to the hydrophilic headgroup), 594neg-SM(18:0) (Hd), 594neg-DSPC (Hd), and 594neg-DOPC (Hd) exhibited two diffusion coefficients in two coexisting domains with higher and lower partitioning (for the first three probes, slower and faster diffusion, respectively; 594neg-DOPC was opposite), and thus 488neg-SM(18:0) (Hd), 594neg-SM(18:0) (Hd), and 594neg-DSPC (Hd) were categorized as Lo-phase preferring probes and 594neg-DOPC (Hd) was categorized as an Ld-phase preferring probe (See the chemical structures of these probes in Fig. 2). Thus, this class of SM and PC probes have become the second cases, where their Lo/Ld preferential partitioning in GUVs (and in GPMVs) was established without depending on the complementary or colocalized staining of another probe.

6. Giant PM vesicles (GPMVs), PM spheres, and blebbed PMs, which lack the actin-based membrane skeleton (MSK): The Lo-phase-like domains are induced at ~10˚C

Although GUVs are useful for advancing our fundamental understanding of Lo-Ld phase separation, which might be related to many PM functions that non-linearly depend on the cholesterol concentration, the extension of such knowledge gained in GUVs toward understanding the events occurring in the PM is not obvious. This is because (1) the mammalian PM molecular composition is much more complicated, containing over 10,000 molecular species (several thousand molecular species of both lipids and proteins[99]) and including the proteins transiently associated with the outer and inner PM surfaces, and also because (2) the mammalian PM is bound by the actin-based membrane skeleton (MSK), which is a part of the cortical actin filaments closely apposed to the PM cytoplasmic surface.[11,100-103]

An experimental paradigm widely employed to avoid the complication from the actin-

based MSK, but still including molecular complexity similar to that of the PM interior, is the giant plasma membrane vesicles (GPMVs)[27,29] or PM spheres (so called when the GPMV-like structure is induced without chemical modifications).[28] They are formed by PM blebbing (in the case of GPMVs, blebbing is followed by cleavage from the rest of the cell), and have protein and lipid compositions similar to those of the PM, but without the actin-based MSK.[104,105] GPMVs are most often prepared from the RBL-2H3 mast cell line, following the report that revived the use of GPMVs,[27,106-108] but they have been produced from other cell lines such as NIH 3T3 cells,[27] HeLa cells,[109] and CHO cells.[53] The CHO-cell PM contains very small amounts of GM1 (but is strongly enriched with GM3), and thus PMs of and GPMVs prepared from CHO cells would be useful as experimental paradigms that hardly contain GM1.

Upon cooling to ~10˚C, GPMVs generally exhibited two coexisting (and complementary) micron-scale domains (greater than the optical diffraction limit of ~200 nm; Fig. 3). The transition from a single-domain to two coexisting domains started occurring at around 25˚C and was nearly complete at ~10˚C.[27-29,109,110] Surprisingly, both of these domains were found to be liquid. First, LRB-1-stearoyl-2-oleoyl-PE was found to diffuse rapidly in both domains with diffusion coefficients of 1.8 and 5.6 $\mu m^2/s$[29] (Table 2), which were quite comparable to those found for fluorescent phospholipid analogs in Lo- and Ld-phase domains in GUVs, respectively[31] (Table 2; although the diffusion coefficient in the slow domain was somewhat larger than that found in the Lo-phase domain in GUVs). The domain with the higher (lower) diffusion coefficient coincided with the domain that was more strongly (weakly) labeled with LRB-1-stearoyl-2-oleoyl-PE. Second, the lipid packing (order) evaluated by C-Laurdan suggested that both were in the liquid state, although the difference in the packing between the two domains was much smaller than that found between Lo- and Ld-phase domains in GUVs[58,108] (the results reported in Kaiser et al.[58] included the data obtained by using both GPMVs and PM spheres). These results were quite surprising when they were initially reported. One would generally expect that GPMV lipids would solidify at lower temperatures, and that before the total solidification eventually occurs in the cooling process, some solid domains would start forming, inducing the coexistence of *solid* and liquid domains[111]. However, it was difficult to predict the creation of two complementary, coexisting *liquid* domains in GPMVs within certain temperature ranges (also see curious results reported in Ref. 112, 113, and 114).

Furthermore, these two coexisting domains were complementarily stained with Lo- and Ld-phase preferring fluorescent probes found in GUVs (see top 9 rows of Table 1). These three results suggest that GPMVs at 37˚C are in the Ld-phase-like state, and upon cooling, another domain resembling the GUV's Lo-phase domain appears, leaving the rest of the membrane still in the Ld-phase-like state. Therefore, in this article, these two domains found in GPMVs at ~10˚C are given the names Lo-phase-like and Ld-phase-like domains or "*Lo-like and Ld-like* domains" for short, respectively, as we do not have any proof to unequivocally show that these domains could thermodynamically be called "phases". The molecular-scale observations of GPMVs using spin-label EPR spectroscopy supported the presence of two domains with distinctively different order parameters for the acyl chains (the order parameter for the higher-order state is intermediate between those of the Lo-phase and Ld-phase domains in GUVs[115]).

These observations are extremely important, as they show that

(1) at lower temperatures, the PM after the removal of the actin-based MSK is capable of undergoing a cooperative process resembling the micron-sized liquid-liquid phase separation found in GUVs with special lipid compositions; therefore, that

(2) the actin-based MSK blocks the formation of micron-sized Lo-like domains upon cooling (also see Refs. 116, 117, and 118 for the coupling of actin filaments and lipid domains), and that

(3) micron-sized domains do not form at the physiological temperature of 37˚C, even in the absence of the actin-based MSK.

7. The presence of >25-mol% overall cholesterol concentrations is essential for the generation of Lo-like domains in GPMVs at lower temperatures

Another critically important observation was made using GPMVs. After mild partial cholesterol depletion, the Lo-phase-like micron-scale domains do not exist even at 10˚C,[109] suggesting that the presence of overall cholesterol concentrations of 25 mol% or more is essential for producing Lo-like domains in GPMVs at ~10˚C. The requirement of >25 mol% overall cholesterol mole fraction to produce Lo-like domains in GPMVs at ~10˚C is pretty much the

same as that for many PM signaling functions, as well as that for the induction of Lo-Ld phase separation in GUVs consisting of the ternary mixtures of DSPC(DPPC)/DOPC/cholesterol.

8. Do nano~meso-scale Lo-like domains exist in the PM at 37˚C?

Considering the observations made with GUVs and GPMVs and simulations based on the Ising model, Veatch, Keller, and their colleagues proposed that the micron-sized Lo-like domains found in GPMVs at 10˚C could become nano-meso-scale domains at 37˚C, due to the critical fluctuations of the lipid compositions that remain when the temperature is raised by 15 - 20 degrees from the temperature of the transition from the two-phase (Lo and Ld) state to a single-phase (Ld) state (miscibility transition temperature T_{misc} ~20˚C).[23,24,81,119-121] It is true that the structural and compositional fluctuations must exist as precursors to the phase separation even above T_{misc}, but the problem is how extensively (in terms of size and number) they remain at 37˚C. Veatch, Keller, and their colleagues concluded that, due to the remaining criticality, spatial variations of the lipid compositions with the correlation length of ~20 nm[120] or ~5 nm[119] in GPMVs could occur, giving rise to Lo-like domains of these sizes at 37˚C.

However, no direct detection or observation of Lo-like domains in GPMVs at 37˚C and PMs (at any temperature) was made. This is probably due to their small sizes of 5 ~ 20 nm. There are other indications suggesting that the Lo-like domains in GPMVs and PMs are this size. We previously determined that TM proteins, many of which are not sterically (laterally) compatible with cholesterol and thus with Lo-like domains, are located ~4 nm apart from each other in the PM,[122] suggesting that the growth of Lo-like domains much greater than 4 nm in diameter in the PM of quiescent cells might be difficult. As described in Section 4, even in GUVs, under certain conditions, the Lo-phase domain sizes could be on the nano-scale.[82] For example, in the presence of POPC and SOPC, which are abundant in the PM and considered to be surface active at the interface between the Lo-phase and Ld-phase domains, the Lo-phase domains of nanometer-sizes (≥5 nm) were formed. This result suggests the possibility that the raft domains in the quiescent-cell PM might have similar sizes.

In the Lo-phase domain in the artificial membranes made of the ternary mixtures of saturated phospholipids/DOPC/cholesterol, the complexes of cholesterol and saturated acyl chains at ratios of 1:2, 1:4, and 1:8 (cholesterol/PC ratios of 1:1, 1:2, and 1:4, respectively),

which are immiscible with unsaturated PC, are likely to form, as reviewed previously[4] and in Section 4. Some of these complexes might remain even when the GUVs are in a single-phase state, perhaps forming greater complexes. If two complexes made of the 1:2 mixture of cholesterol and saturated acyl chains (1:1 mixture of cholesterol and a saturated phospholipid, and thus 4 molecules) were to form a greater complex (to define raft domains, we would need at least three molecules in the domain for cooperative interactions), then it would occupy an area of ~2 nm in diameter, which would be the smallest possibility. Taken together, the sizes of the Lo-like domains in GPMVs and PMs, without considering the presence of the actin-based MSK, in quiescent cells might be between 2 and 20 nm. As such, the problem of whether nano~meso-scale Lo-like domains exist in the PM in quiescent cells at 37˚C is the most speculative issue in raft domain research (see the item 5 in the next section).

9. Working definition of raft domains

First, we will summarize the results obtained by using the GUVs and GPMVs described in the previous sections into the following five key points:

(1) the non-linear cooperative dependence of various PM functions on the cholesterol concentration in the PM, with typically full function at the physiological ~40 mol% cholesterol and virtually no function below 25 mol% cholesterol,

(2) the existence of the Lo-phase domain in GUVs made of the ternary mixtures of saturated phospholipid/DOPC/cholesterol at cholesterol concentrations higher than 25 mol%, which are readily observable below 25˚C and detectable even at 37˚C, at limited mole fractions of the three components, and

(3) the cholesterol-dependent appearance of the complementary two micron-scale domains in GPMVs at ~10 ˚C (which starts below 25˚C upon cooling from 37˚C; micron-scale domains appear only after the removal of the actin-based membrane skeleton bound to the PM cytoplasmic surface; *i.e.*, upon the production of GPMVs), which suggests the occurrences of nano~meso-scale (2-20 nm) Lo-like domains at temperatures above 25˚C, such as 37˚C, as expected from both theory and simulation.

In addition, we include the following two additional results in considering the putative raft domains that exist in GPMVs and intact PMs at 37˚C:

(4) the complexes of cholesterol and saturated acyl chains at ratios of 1:2 and 1:4 (cholesterol/PC ratios of 1:1 and 1:2, respectively), which are immiscible with unsaturated PC, are likely to be the basic units for forming Lo domains in the ternary mixtures of saturated PC/cholesterol/DOPC,[4] and

(5) GPI-anchored proteins form metastable homodimers with a lifetime of ~160 ms (in the case of CD59) in the PM, due to the protein-protein interactions between the extracellular protein domains. The homodimer lifetime was shortened by a factor of 2, when cholesterol was partially depleted, even though ~25 mol% cholesterol still remained in the PM. A reduction of the homodimer lifetime by a factor of 2 was also observed when the GPI-anchoring chains were replaced by the TM domain of LDL receptor; *i.e.*, the interactions with 25 and 0 mol% cholesterol gave the same effect (*i.e.*, no effect prolonging the homodimer lifetimes). These results indicate that the homodimers of GPI-anchored proteins are stabilized by the interactions with the cholesterol-enriched domains formed in the presence of >25 mol% cholesterol, implying the cooperative interactions of the GPI-anchoring chains (saturated acyl chains) and the cholesterol-enriched complexes/domains.[43] (The readers might correctly raise a question about the appropriateness of citing this one paper here as an evidence in support of the existence of raft domains in the PM in living cells at 37˚C, but we cannot find any other reports directly showing the presence of molecular assemblies depending on cholesterol concentrations in cooperative ways in the PM in quiescent cells, in line with the item (1), and here we need such papers, or at least a paper, that indicate, in the PM, the existence of molecular assemblies whose existence nonlinearly depends on cholesterol concentrations, for indicating the presence of such molecular assemblies in the PM. Therefore, we remind the readers of being aware that there could be pros and cons about whether this type of result is taken as a strong argument for the existence of raft domains in the PM.)

Based on these five observations, we propose the following working definition of raft domains in the PM. "*Raft domains in the PM are liquid-like molecular complexes/domains formed by cooperative interactions of cholesterol with saturated acyl chains as well as unsaturated acyl chains, due to saturated acyl chains' weak multiple accommodating interactions with cholesterol and cholesterol's low miscibility with unsaturated acyl chains and TM proteins (collectively called, 'raft-lipid interactions')".* How such raft domains in the PM can

be detected will be described in Sections 10 and 14. In the following part of this review, the word, "raft domain" indicates the membrane domains defined here. Since, in this definition, the raft domains in the PM are basically composed of cholesterol and saturated acyl chains, we call these raft domains "C-SAC rafts", when the meaning of the "raft domain" may not be clear.

Raft domains are in the liquid state, because, as shown in GUVs, the domains consisting of cholesterol and saturated acyl chains, generated as defined in this working definition, are known to be in the liquid state. The "liquid state" means that (1) cholesterol and molecules with saturated chains and/or the basic complexes for generating the raft domains (1:1 and 1:2 complexes of cholesterol and molecules with two saturated acyl chains) can physically exchange their positions within the complex, (2) they can exchange with those located in the bulk Ld-like domain, and (3) raft domains can coalesce to form larger domains by suitable triggers.

Since such liquid-like domains would only form in the presence of >25 mol% cholesterol (typically ~40 mol% cholesterol) at 37˚C in a membrane containing both saturated lipids and unsaturated lipids, this working definition is consistent with all of the Lo-phase and Lo-like domains found in GUVs and GPMVs. Indeed, this working definition was made possible by integrating our knowledge obtained by using GUVs, GPMVs, DRM/non-DRMs, and PMs.

More specifically, the following three points are the key for this definition.

(1) >25 Mol% cholesterol is an essential component for creating raft domains (in the mammalian PM).

(2) Cooperative assembly of the complex of cholesterol and saturated acyl chain (often 1:1 and 1:2 complexes of cholesterol and lipid; the ratios of cholesterol vs. saturated acyl chains are 1:2 and 1:4, respectively) is the first key for raft formation.

(3) Cooperative exclusion of unsaturated chains from raft domains due to immiscibility of the unsaturated chains with cholesterol is the second key for raft formation.

For the details of the second and third molecular interactions, see our previous review published in *Traffic*.[4] We propose that the second and third molecular interactions described here induce the cooperative formation of Lo-phase-like domains in the PM, which one could call phase separation in a broader sense. Since this definition is based on the results obtained in GUVs, GPMVs, DRMs, and PMs (although the data obtained in the PM directly showing the presence of metastable nano-meso-scale raft domains are limited to only a few pieces of single-

molecule imaging results[30,31,43]), all the properties of raft domains, such as those described at the beginning of this review, should be able to be derived or at least qualitatively explained by this definition.

One of the strongest pieces of experimental evidence for the existence of the raft domains *in the intact PM* will be the presence of the homodimers of GPI-anchored proteins, with lifetimes prolonged by their cooperative interactions with cholesterol, as stated in point 5 for defining the raft domain. One of the weakest points for this working definition is that the definition is only qualitative. So, the next step to improve this working definition is to make the description more quantitative. We could consider the raft domains in the PM of non-stimulated cells as metastable liquid-like molecular complexes/domains of cholesterol and saturated acyl chains, formed due to their cooperative multiple molecular interactions and the immiscibility of cholesterol with unsaturated acyl chains, as described in the working definition, and/or due to critical lipid-compositional fluctuations, as described in the previous section (Section 8). These statements probably represent different ways of saying the same thing, with an emphasis on different aspects (however, see Ref. 123). The latter view is a definition from the statistical physics viewpoint, and is more quantitative.

Why is it useful for the cells to maintain nano~meso-scale raft domains in the PM before the extracellular signals or other cues arrive? After the arrival of the extracellular signals, larger and more stable signaling raft domains might form simply from the homogeneous PM, in the absence of pre-existing metastable raft domains in quiescent cells. Perhaps, the aft domains in resting cells might simply exist without particular functions. One of the strongest pieces of evidence that the nano~meso-scale raft domains existing in quiescent cells play important roles in signaling is again given by the existence of the GPI-anchored protein homodimers (as stated in point 5 for defining the raft domain). The pre-existence of the GPI-anchored receptor homodimers in resting cells was found to accelerate and enhance the downstream signaling (more specifically, Ca^{2+} mobilization), and, as stated, the GPI-anchored receptor homodimers are enhanced (their lifetimes are prolonged) by their cooperative interactions with metastable raft domains (see Fig. 7c in Ref. 43).

Five major mysteries remain, regarding the metastable nano~meso-scale raft domains in the PM. First, the cholesterol distribution in the PM outer and inner leaflets is controversial.

As much as 60-70% or only 8% of the PM sterol might exist in the cytoplasmic leaflet.[75,124] This means that cholesterol constitutes approximately 40 mol% or 5 mol% of the cytoplasmic leaflet lipids, respectively. In this review, we assumed that at least 50 % cholesterol exists in the PM outer leaflet, based on the traditional view of the rapid flip-flop of cholesterol.[125] More efforts should be made for developing cholesterol-binding probes and cholesterol-analog probes that mimic the behaviors of cholesterol in the PM, such as those described in Refs. 75, 124, and 126.

Second, related to the first mystery, the means by which the raft domains or Lo-like domains in the PM outer leaflet are coupled to those in the PM inner leaflet are unknown.[127,128] This has been a major issue in the field of lipid-raft research, and it was covered in detail in our previous review in this journal, published 15 years ago.[4] Recently, Raghupathy et al. found that transbilayer interactions between artificial GPI-anchored proteins with long acyl chains and inner-leaflet phosphatidylserine are pivotal in generating actin-dependent nanoclusters of the GPI-anchored protein, which might be one of the major mechanisms for the generation of lipid raft domains.[129] Nevertheless, further major advancement in our understanding of the interbilayer coupling will be needed.

Third, the lifetimes of the raft domains in quiescent cells are totally unknown. The stabilized raft domains generated for signaling by the engaged, clustered GPI-anchored receptors appear to exist quite stably for minutes to 10s of minutes. Meanwhile, the dwell times of raftophilic lipid probe molecules and signaling molecules in a raft domain might be on the order of several 10s of milliseconds.[30,31,43] Therefore, we suspect that the raft domain lifetimes in quiescent cells might be on the order of 0.1 to 10 s, but they have never been measured. The dwell lifetimes of raftophilic lipid probes in both metastable raft domains in quiescent cells and stabilized raft domains in stimulated cells should also be clarified. In Section 2, as one of the two reasons why defining raft domains is difficult, we raised the point that the raft domains in quiescent cells are difficult to visualize, due to their small sizes (2-20 nm). Here, we would like to point out that they are also difficult to visualize due to their shorter lifetimes, on the order of 0.1 to 10 s.

Fourth, how the compositions of cholesterol and saturated and unsaturated lipids in the PM are regulated, including the lipid sensors and trafficking, is unknown.[127-129]

Fifth, understanding the formation and function of raft domains would not be complete without incorporating the notions of three-dimensional membrane curvatures and shapes, including the involvement of curvature driving proteins. The research in this direction is quite important, but it appears to be still in the fledgling stage.[130-132]

10. Fluorescent lipid probes that might be useful for raft-domain research

The molecular compositions of GPMVs would be very similar to those of cellular PMs, and therefore, in this section, we pay more attention to GPMVs than GUVs. The purpose in this section is to establish the criteria for fluorescent lipid probes that will preferentially partition into the (C-SAC) raft domains and non-raft domains in the PM.

The Lo-like and Ld-like domains in GPMVs have been most often defined by using two fluorescent lipid probes that exhibited complementary staining, rather than by observing the diffusion or acyl-chain order (lipid packing) in each domain. This sometimes created a logical problem as well as incorrect interpretations of the results in the GPMV literature. The probe's preference for Lo- or Ld-like domains was often not proved independently, and therefore, the premises were often justified by the conclusions, just like the cases found in the GUV literature. For example, fluorescent lipid probes anchored to the membrane by way of two palmitoyl chains or those preferentially partitioning into the Lo-phase domain in GUVs were (often wrongly) assumed to be raft-associated and used to observe presumable raft domains in the PM, without really testing their partitioning into Lo- or Ld-like domains in GPMVs (see Table 1). These are basically the same as making the statement, "the raft domain was found by a probe that was assumed to partition into the raft domain"; *i.e.*, "all of the domains found by this probe are raft domains because we assumed that it is a raft-associated probe". Before using the probe, it should have been independently shown that the probe actually does partition into the raft domain (or non-raft domain), but this was often not properly performed in the lipid-raft research field. This is probably because the lipid-raft literature has been quite confusing and full of such statements. Here, we reorganize the results in the literature, and find the logically well-defined fluorescent lipid probes.

See the first row and the column of GPMVs in Table 1. Kaiser et al.[58] found that, in GPMVs exhibiting the presence of two complementary micron-scale domains after labeling with LRB-DOPE and C-Laurdan, the domains more intensely labeled with LRB-DOPE are colocalized with C-Laurdan's lower polarity region (more disordered domain), which establishes that LRB-DOPE is an Ld-like-domain-preferring probe in GPMVs (just as is the case with GUVs). Starting from this, we determine whether a test probe A (the first one is the CTB-GM1 pentamer complex; see the second row and the column of GPMVs in Table 1) preferentially partitions in a complementary manner with LRB-DOPE in GPMVs. Such complementary staining and quite homogeneous appearances of each domain in GPMVs are highly valued, because the creation of two complementary liquid domains (rather than >10,000 domains) is not guaranteed in GPMVs, according to the thermodynamic principles (phase rules) (see details in the caption/note to Table 1).

If the preferential association of probe A is determined, then the preference of another probe B can then be determined by complementary staining (and/or colocalized staining) of LRB-DOPE and/or probe A in GPMVs, as was done for the GUV data. We examined this by finding images in the literature, and the results are summarized in Table 1 (Therefore, when Table 1 is read for the first time, it should be read from the top row toward the bottom row).

More specifically, we performed the following analysis (Table 1). After establishing (finding) that the LRB-DOPE-concentrated domains in GUVs and GPMVs that exhibited the existence of two complementary micron-scale domains were Ld-like domains,[58] we then turned to PM spheres (which are quite similar to GPMVs) labeled with LRB-DOPE and then treated with CTB. In the PM spheres, the CTB-concentrated domains were formed, and the CTB domains were found (by the original authors) to exist as domains complementary to the LRB-DOPE domains in both GUVs and GPMVs, and to be colocalized by C-Laurdan's higher polarity domains in GPMVs, showing that the CTB domain is in the Lo-like state.[27] This was further confirmed by the observations in which the PM spheres treated with CTB exhibited the existence of two diffusion coefficients of CTB-Alexa Fluor 488 (0.13 and 1.3 $\mu m^2/s$, comparable to the diffusion coefficients in the liquid domains), independently showing the presence (creation) of Lo- and Ld-like domains after the addition of CTB to PM spheres.[28] We continue such evaluations down to the end of Table 1.

Finally, we define the criteria for fluorescent lipid probes that will preferentially partition into the raft domains. Among the five key points used to define raft domains (Section 9), the points relevant for defining the lipid probes are the following three points.

(2) preferential partitioning into the Lo-phase domains in liquid-liquid phase-separated GUVs,

(3) preferential partitioning into Lo-like domains in GPMVs at lower temperatures such as ~10˚C

(5) partitioning into (transient recruitment to) GPI-anchored proteins' metastable homodimer rafts in the PM.[43]

The fifth point is quite difficult to test for all the relevant lipid probes because the test requires simultaneous two-color, single-molecule imaging and tracking. As a replacement, we could examine the extent of partitioning of the lipid probes into antibody-induced GPI-anchored protein patches (due to space limitations, we cannot include an explanation of this method. Readers are referred to Fig. 3 and Supplementary Figs. 3-5 and their captions in Ref. 30), but such examination of fluorescent lipid analogs are quite rare, and not suitable to employ for our search in literature here. However, it was shown in Ref. 30 that, although the number of examples are limited, the preferential partitioning of the lipid probes into antibody-induced GPI-anchored protein patches consistently occur with the probes' partitioning into detergent-resistant membranes (DRMs) after the probes' incorporation into the live-cell PM and the subsequent treatment of the cells with the detergent Triton X-100 at 4˚C (the ratio of the signal intensities after vs. before the addition of Triton X-100 at 4˚C).

Therefore, we propose to define the fluorescent lipid analogs that preferentially partition into raft domains (non-raft domains) in the PM as those molecules that satisfy all of the following three criteria.

(1) preferential partitioning into Lo-like domains (Ld-like domains) in GPMVs at lower temperatures such as ~10˚C;

(2) preferential partitioning into the Lo-phase domains (Ld-phase domains) in liquid-liquid phase-separated GUVs; and

(3) preferential partitioning into DRMs (non-DRMs) after its incorporation into the live-cell PM and the subsequent treatment of the cells with the detergent Triton X-100 at 4˚C.

In the strict sense, the DRMs must be the specimens obtained by using sucrose density-gradient fractionation of the cold-Triton-treated cells, rather than just the material that was not dissolved in cold Triton X-100, because, for example, proteins bound to the cytoskeleton, without any relevance to raft domains, might not be dissolved in Triton X-100. However, since most lipid probes would not bind to the cytoskeleton, the domains marked by the fluorescent lipid probes in the PM and remaining after the cold-Triton treatment would form lighter fractions after density-gradient fractionation, and hence we would consider the domains remaining after the cold-Triton treatment to be the DRMs. This justifies the third criterion.

In the biochemical literature and early raft-domain studies, DRMs were often called "lipid rafts", which caused unnecessary confusion. Since DRMs are not lipid rafts in the PM, DRMs should simply be called DRMs, and not lipid rafts.[15,47,49] The results shown in the column of DRMs in Table 1 should be read with this important understanding.

Based on these three criteria, fluorescent lipid probes for raft or non-raft domains in the PM are summarized in the far-right column in Table 1. From the results summarized in Table 1, the following sphingomyelin and phosphatidylcholine molecules are selected as logically and practically excellent markers for raft domains in the PM: 488neg-SM(18:0) (Hd), 594neg-SM(18:0) (Hd), 594neg-DSPC (Hd), and 488neg-DSPC (Hd) (CTB could create Lo-like domains, and thus would not be useful as a probe. However, this would not affect its valuable ability to test the Lo/Ld preferences of other probe candidates). These lipid analog probes exhibited two different diffusion coefficients in Lo- and Ld-like domains in GUVs or GPMVs (Lo-like domains were more densely stained by a factor of approximately four; Table 2), confirming their preferred domains and the liquid natures of both domains, which further show that they are excellent probes. The common structures of these probes are the presence of a saturated long acyl chain (C18:0) and a fluorescent probe moiety placed far away from the membrane via the hydrophilic "neg" linker on their headgroups (without losing the positive charge of the choline group; cf. the chemical structures in Fig. 2). Blocking the insertion of the fluorescent probe into the membrane near its parental molecule appeared important particularly for their association with raft domains. Meanwhile, one should not oversimplify or be misled by the term, marker for the raft domain. The preferences of these lipid probes for Lo- and Ld-like domains, particularly Lo-preferring molecules that closely resemble their parental lipids, are not very

strong. For many marker molecules, the partitioning ratios between Lo/Ld (or Ld/Lo) domains in GUVs and GPMVs are often ~4:1[31] (see Table 4 discussed later).

The following molecules were selected as logically and practically excellent non-raft domain markers: all of the fluorescent phospholipids and DiI- and DiO-type probes containing two oleoyl or linoleoyl chains examined thus far (including LRB-DOPE, ATTO647N-DOPE, ATTO594-DOPE, 594neg-DOPC, DiI-C18:1, and DiI-C18:2 [FastDiI], as well as related compounds of DiO), DPPC-C5-BodipyFL (Acyl; the probe attachment to an acyl chain), and 594neg-DOPC. The common feature of these probe molecules is that their native cis-unsaturated acyl chains and fluorescent probes attached to the acyl chains disturb the molecule's interaction with the planar rigid ring structure of cholesterol,[4] and thus will drive these molecules away from raft domains into non-raft domains. However, note that the bulk (Ld-like) domain in the PM would contain lower but substantial concentrations of cholesterol (probably 10~20 mol%), and likewise, the raft domains might also contain unsaturated lipids and TM proteins. Furthermore, some TM receptors might be preferentially located at the interface between the Lo- and Ld-like domains[6,30,133] (see Section 12).

Note that the determinations here are qualitative, and the levels of preferences might vary considerably as described above. More quantitative evaluations are summarized in Table 4, and will be discussed later in this review to establish additional useful fluorescent lipid probes.

11. Proteins that preferentially partition into raft and non-raft domains in the PM

Since the molecular compositions of GPMVs would be very similar to those of cellular PMs and much more so than those of GUVs, here we pay more attention to GPMVs. The preferential partitioning of several proteins (and two gangliosides) in Lo/Ld-like domains in GPMVs (first criterion) shown thus far is summarized in Table 3, after reexamining the literature based on the Lo/Ld-like-domain markers found in the process of producing Table 1; *i.e.*, CTB, Fast DiI, Fast DiO, TexasRed-DPPE, and LRB-DOPE (Table 1). We would also keep the third criterion: partitioning into DRM/non-DRM.

However, we give up the second criterion in some cases; preferential partitioning into

Lo (Ld)-phase domains in phase-separated GUVs, due to the following two reasons. First, there have not been many studies in which membrane proteins are reconstituted into GUVs. Second, as pointed out earlier in this review, simply extending the similarity of the GUVs' Lo-phase domains to Lo-phase-like domains in GPMVs and raft domains in the PM is problematic: the Lo-phase domains in GUVs are much more ordered and less fluid than the Lo-like domains in GPMVs, and probably due to this reason, GPI-anchored proteins, CD59, and Thy-1, as well as EGFP-GPI, partition more into *Ld*-phase domains in GUVs (see Note 3 in Table 3 for further explanations). These are prototypical raft-associated proteins, and we rather break the second criterion, which has its own limitations, than categorizing them into proteins that equally partition into both raft domains and non-raft domains in the PM. However, we emphasize again that their preferences of the partitioning into raft domains and non-raft domains are quantitative and not all-or-none. Minor but substantial fractions of GPI-anchored proteins are likely to exist in non-raft domains in the PM.

Transferrin receptor (constitutive covalent dimer containing two TM domains) and high-affinity IgE receptor (αβγ2 tetramer containing five TM domains) before stimulation were found to be good markers for the non-raft domains in the PM. For the structural determinants of TM proteins that partition into raft domains, which we do not address in this review, readers are referred to the marvelous paper by Lorent and colleagues.[14] Palmitoylation was concluded to be one of the critical conditions for incorporation of TM proteins into raft domains.[14]

Hayashi's group recently proposed a new interesting mechanism for incorporation of TM proteins linked by palmitoyl chains into cholesterol-enriched domains.[134,135] Rhodopsin, a GPCR with seven TM domains, has two covalently-linked palmitoyl chains, but only when they form dimers, they are likely to associate with cholesterol-enriched domains. They proposed that this probably occurs because the hydrophobic surface of the dimer is better covered with palmitate than that of the monomer (and possibly with the smoother parts of the 7 TM domains). Such a mechanism for the association of TM proteins with raft domains based on dimerization-multimerization-induced changes of the hydrophobic surfaces of TM proteins is totally new and very appealing.

12. Some TM receptors might function at the interface between the raft

and bulk domains

A high-affinity IgE-Fc receptor (FcεR) consists of four subunits containing a total of five TM domains, and is an Ld-preferring molecule in GPMVs. It does not recruit fluorescent SM analogs or raft-associated Lyn kinase in the PMs of resting cells. However, after its ligation, it recruits the fluorescent SM and raft-associated Lyn kinase, suggesting that it becomes more associable with raft domains.[31] FcεR might be located at the interface between the raft domain and the bulk domain, which might enable the receptor to adjust its association with both the raft and bulk domains under different conditions.[38,64] Similar Janus-faced associations might occur with other receptors, such as T-cell receptor, B-cell receptor, and EGFR.[136]

The delicate balance of associations with raft/non-raft domains might be a reason for the apparently inconsistent results in the literature, with regard to the Lo/Ld-DRM/non-DRM associations of these receptors.[32,137-141] Their downstream signals are largely suppressed after partial cholesterol depletion to less than ~25 mol% overall cholesterol concentrations, indicating the requirement of raft-lipid interactions for inducing signaling. Note that the actin-based MSK might also be involved in either suppressing unwanted stimulation or enhancing stimulation in the signaling pathways involving raft domains.[32,38,139,142,143]

Meanwhile, upon the cell entry of HIV, CCR5 (a GPCR that can be palmitoylated) preferentially sequestered at the Lo/Ld domain boundaries plays a pivotal role as a co-receptor, together with the HIIV receptor CD4 substantially localized in the Lo-like domains.[133] This was demonstrated using GPMVs derived from HeLa cells that stably express the CD4 receptor and CCR5 co-receptor ($CD4^+/CCR5^+$) (domain separation induced at ≈22˚C). The fusion of HIV with the PM occurs at the boundaries rather than the Lo-like domains.

It would be interesting to understand the general structural rules for the incorporation of TM proteins into the interface between the raft domain and the bulk domain, in addition to the general rules for their partitioning into raft domains (Lo-like domains in GPMVs) as reported previously.[14]

13. Methods for evaluating the involvement of a protein/lipid of interest in raft domains in the PM

To evaluate the involvement of a protein or a lipid of interest in raft domains in the PM, we propose to use the same three criteria employed for defining the fluorescent lipid analogs that preferentially partition into raft domains (or non-raft domains) in the PM:

(1) preferential partitioning into Lo-like domains (Ld-like domains) in GPMVs at lower temperatures such as ~10°C;

(2) preferential partitioning into the Lo-phase domains (Ld-phase domains) in liquid-liquid phase-separated GUVs; and

(3) preferential partitioning into DRMs (non-DRMs) after its incorporation into the live-cell PM and the subsequent treatment of the cells with the detergent Triton X-100 at 4°C.

See Tables 1, 3, and 4. DRM (non-DRM) partitioning coincides with partitioning into Lo (Ld)-like domains in GPMVs quite well, classifying these molecules into the category of raft-associated. However, also note interesting exceptions of several lipid-anchored signaling molecules; *e.g.*, EGFP-Lck-anchor, Lck, EGFP-Fyn-anchor, Fyn, EGFP-H-Ras anchor, and H-Ras. These molecules have been often considered raft-domain associated, but their behaviors in GPMVs are quite complicated.

Nevertheless, the results shown in these tables suggest that, in the preliminary testing of a molecule's possible association with raft domains in the PM, examining whether the molecule partitions into DRMs after the cold-Triton treatment would be a good starting point.

Therefore, when evaluating a protein or a lipid of interest for its possible association with raft domains, we recommend the following protocol (in the order of experimental ease).

1) Examine the extent of partitioning into DRMs.

2) If the molecule is present or can be expressed in cells in culture, examine the extent of colocalization with antibody-induced GPI-anchored protein patches (due to space limitations, we cannot include an explanation of this method. Readers are referred to Fig. 3 and Supplementary Figs. 3-5 and their captions in Ref. 30). If possible, observing transient associations with the metastable homodimer rafts of GPI-anchored proteins is better.

3) Prepare GPMVs and examine the extent of partitioning into the Lo-like domain at ~10°C. Make sure to use the marker molecules established here for both Lo-like and Ld-like domains (which should stain the GPMVs complementarily).

4) If the molecule can be reconstituted in GUVs, then examine the extent of partitioning

into the Lo-phase domain. The temperature could be lowered to 15 - 25˚C for easier detection of the Lo-phase domains. As described, note that some molecules partition into Lo-like domains in GPMVs, but into Ld-phase domains in GUVs. If this occurs, the GPMV result is more reliable for judging the raft association of the molecule, because the molecular compositions of GPMVs resemble those of the PMs much more than those of the GUVs.

14. Additional useful lipid probes for studies of raft domains in the PM (Table 4)

Table 1 was organized by following the process for logically defining raft-associated probes and domains. Table 4 provides *quantitative* evaluations of the partitioning of fluorescent probes into Lo-/Ld-phase domains in phase-separated GUVs, Lo-/Ld-like domains in GPMVs at 10˚C, and DRMs vs. intact-PM (T24 cells). In addition, *qualitative* data for the colocalization of each probe with CD59 patches (item 3 in the previous section) are included.

In Table 4, the various lipid probes are grouped based on the lipid types. The major additions here are the new fluorescent ganglioside analogs, including the analogs of GM1, GM2, GM3, and GD1b (their chemical structures are shown in Fig. 4). Since these probes are classified into the "raft-domain-associated" category, they would be quite useful in raft-domain research.

In this review, we left out most of the peptide/protein probes that have been used to detect specific lipids in the plasma membrane.[144-149] They have revealed important properties of raft domains, but since their binding to specific lipids is often very sensitive to particular lipid-lipid interactions and also to masking by the binding of their physiological binding partner proteins.[145,146,150-153] Therefore, these results were difficult to use for defining raft domains. But their sensitivities for lipid-lipid and protein-lipid interactions themselves are particularly useful for studying various raft properties and functions and special types of raft domains in the PM, based on their special sensitivities to specific molecular interactions.[151-153]

15. Concluding Remarks

In this review, we have focused our efforts on considering and reorganizing the existing data in the literature in a logical manner, to provide a working definition of raft domains in the PM. These domains are formed by the cooperative interactions of cholesterol with saturated and

unsaturated acyl chains in the PM. The assembly occurs cooperatively, because the raft domains disappear when the cholesterol concentration in the PM is decreased from ~40 mol% to <25 mol%. Under these conditions, many PM functions are lost, despite the presence of relatively high concentrations of cholesterol. The raft domains are likely to exist on the ~nano-meso scale in quiescent cells, and could be coalesced to form larger, stabilized domains after the reception of certain extracellular signals, for downstream signaling.

In the process of examining the data in the literature, we extensively depended on the results obtained with fluorescent lipid analogs. We first found that LRB-DOPE has been *independently* shown to partition into the Ld-phase domains in GUVs, and the Ld-like domains in GPMVs. Starting from these observations, we were able to reorganize the lipid partitioning data, without being trapped in the circular logic, by examining the complementary and colocalized partitioning with respect to the lipid probes with established partitioning behaviors in a logical order (Table 1).

We proposed the following three criteria for the expected association of fluorescent lipid probes with raft or non-raft domains. They are: (1) the Lo/Ld preferences in GPMVs, (2) consistency in the probe's Lo/Ld partitioning preferences in liquid-liquid-phase-separated GUVs, and (3) consistency in the probe's partitioning into DRMs.

Particularly, we provided a collection of the fluorescent lipid probes that have been logically established to preferentially partition into the raft and non-raft domains in the PM, based on these three criteria. Since these fluorescent lipid probes are shown here to satisfy the three criteria, they can be used for future studies without reservation and without the concern of being trapped in the circular logic. We hope the lists of these probes, Table 1 and Table 4, are useful for future raft-domain research.

As the readers are warned in Introduction, the end results, the definitions of raft domains and the list of useful lipid probes for raft-domain research in the PM are probably not very different from what many scientists in the field anticipated. However, we believe that the raft-research field has not had a clear simple definition of raft domains in the PM before, and the field now needs this kind of working definition badly for its healthy development. The definition should be made so that all the properties of raft domains, such as those described at the beginning of this review, could be derived or at least qualitatively explained by this definition.

Since we formulated the proposed working definition based on, in principle, all the results obtained in GUVs, GPMVs, DRMs, and PMs (although the data obtained in the PM directly showing the presence of metastable nano-meso-scale raft domains are limited to only a few pieces of single-molecule imaging results[30,31,43]), the working definition developed here would satisfy the criteria to be called a definition. This definition emphasizes cooperative interactions of cholesterol with saturated acyl chains as well as unsaturated acyl chains, due to saturated acyl chains' weak multiple accommodating interactions with cholesterol and cholesterol's low miscibility with unsaturated acyl chains and TM proteins, i.e., cooperativity including the exclusion of unsaturated chains from the neighborhood of cholesterol and the exclusion of cholesterol from the neighborhood of unsaturated chains.

The fluorescent lipid probes recommended in Table 1 and Table 4, can now be used for future studies without reservation and without the concern of being trapped in the circular logic. They could be used as true bona-fide markers for the raft and non-raft domains in the PM.

Despite our efforts, we probably have overlooked other fluorescent lipid probes that could have been included in these lists of logically established probes, because we probably missed many important publications. We apologize to the authors for our oversight.

Acknowledgements

We thank Prof. Makoto Kiso of the Department of Applied Bioorganic Chemistry, Gifu University and the Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, and Prof. Michio Murata of Project Research Center for Fundamental Science and Department of Chemistry, Graduate School of Science, Osaka University, for long-term collaborations, fruitful discussions, and encouragement, which led to this review article.

Table 1. Fluorescent lipid probes examined for their association with raft/non-raft domains in the PM, based on their partitioning into two coexisting liquid domains in GUVs and GPMVs as well as into DRMs/non-DRMs after cold-Triton treatment, arranged so that the examination process is clear from the top row to the bottom row.

When this table is first read, it should not be read randomly, but should be read from the top to bottom to avoid circular reasoning (see the main text). Note that the preferences of Lo/Ld-phase domains in GUVs, Lo/Ld-like domains in GPMVs (at ~10˚C), and DRMs/non-DRMs are largely quantitative. The raft domain in this table is that defined in Section 9 (cholesterol-saturated acyl chain [C-SAC]-raft). The judgement on whether the lipid probes are associative with raft or non-raft domains was made quite conservatively so that the obtained results are robust. The probes with possible complications were not recommended here for the general use for the research of raft domains in the PM. Readers are encouraged to also refer to Ref. 55. Note that all these probes partition into both domains, and the level of partitioning would vary greatly from probe to probe even for probes classified into the same category (see Table 4 for more quantitative data).

The names of the fluorescent lipid analogs are given depending on where the fluorophore is attached. For analogs with the fluorophore attached to the head group, the fluorophore name is followed by the name of the parent molecule. For analogs with the fluorophore attached to the acyl chain, the name of the parent molecule is followed by the probe name.

Probes	Preferences in GUVs[a]	Refs	Preferences in GPMVs at ~10˚C[a] and molecules examined for complementary/ colocalization staining	Refs	Partitioning in DRMs compared with the intact PM at ≤4˚C[b]	Refs	Expected association with rafts or non-rafts[c]
LRB-DOPE[d]	Ld-phase domain: LRB-DOPE domains colocalized by C-Laurdan's lower polarity domains	58	Ld-like domain: LRB-DOPE domains colocalized by C-Laurdan's lower polarity domains	58	non-DRM	30 31	Non-raft
All the phospholipid probes,	Ld-phase domain	25	Ld-like domain	30			To avoid

DiI probes, and DiO probes containing two oleoyl or linoleoyl chains, including ATTO647N-DOPE, ATTO594-DOPE, DiI-C18:1, DiI-C18:2 (FastDiI), DiO-C18:3 [Fast DiO][e] as well as DPPC-C5-BodipyFL (Acyl)[f] (with large hydrophobic probe linked to an acyl chain): based on reasonable *speculations* from the results shown in Ref. 25, 30, and [104]	(clear images) (DiI-C18:1, DiO-C18:3 [Fast DiO])		(clear images) DPPC-C5-BodipyFL (Acyl), ATTO647N-DOPE, and ATTO594-DOPE were clearly colocalized	104 30			confusion by the name of DPPC, we would not recommend using DPPC-C5-BodipyFL (Acyl) as a probe, although we used it previously[30,31]
GM1 + CTB (pentameric GM1 induced by CTB)	Lo-phase domain: Complementary to LRB-DOPE and DiO	58	Lo-like domain: Two diffusion coefficients of CTB-Alexa Fluor 488 in PM spheres (0.13 and 1.3 $\mu m^2/s$), independently showing the presence (creation) of Lo- and Ld-like domains, respectively Colocalized by C-Laurdan's higher polarity domain Complementary to LRB-DOPE Complementary to Fast DiO Monomeric CTB slightly preferred partitioning into the Ld-like domain[g]	28 58 27 58 104 108 123	DRM DRM[h] (native GM1 without CTB)	29 104 154 155 156	Raft
488neg-SM(18:0) (Hd)[i,j]	Lo-phase domain (Lo/Ld = 4.1 ± 0.15):[k] Two diffusion coefficients (0.9 and 5.5 $\mu m^2/s$), independently showing the presence of Lo- and Ld-phase domains	31 31	Lo-like domain (Lo/Ld = 4.8 ± 0.50): Complementary to DPPC-C5-BodipyFL (Acyl)	31	DRM	31	Raft
594neg-SM(18:0) (Hd)[j]	Lo-phase domain (Lo/Ld = 3.9 ± 0.15):[k] Two diffusion coefficients (0.9 and 5.4 $\mu m^2/s$), independently showing the presence of Lo- and Ld-phase domains	31 31	Lo-like domain (Lo/Ld = 4.7 ± 0.50): Complementary to DPPC-C5-BodipyFL (Acyl)	31	DRM	31	Raft
594neg-DSPC (Hd)[l]	Lo-phase domain (Lo/Ld = 4.3 ± 0.24): Two diffusion	31 31	Lo-like domain (Lo/Ld = 4.5 ± 0.40): Clear colocalization	31 31	DRM	31	Very likely to be Raft[m]

	coefficients (0.95 and 5.2 $\mu m^2/s$), independently showing the presence of Lo- and Ld-phase domains		with 488neg-SM, but no experiments showing complementary staining with Ld-preferring probes				
488neg-DSPC (Hd)[l]	N.D.[n]		N.D.		DRM	31	Very likely to be Raft[o]
594neg-DOPC (Hd)[l]	Ld-phase domain (Lo/Ld = 0.14 ± 0.01):	31	Ld-like domain (Lo/Ld = 0.20 ± 0.02):	31	non DRM	31	Non-raft
	Two diffusion coefficients (0.9 and 6.0 $\mu m^2/s$), independently showing the presence of Lo- and Ld-phase domains	31	Complementary to 488neg-SM	31			
TexasRed-DPPE (Hd)[p] LRB-DPPE (Hd)[p] FITC-DPPE (Hd)[p]	Ld-phase domain (clear images):	23	Ld-like domain (clear images):	104	N.D.		Better to use probes containing dioleoyl chains, to avoid any confusions[q]
	FITC-DPPE monomers complementary with CTB,	157	Clear colocalization with LRB-DOPE				
	but crosslinked FITC-DPPE became colocalized with CTB (supported monolayers)	158					
diI-C16:0[r]	Ld-phase domain (clear images)	25	Ld-like domain:	27	N.D.		Better to use diI-C18:1 and diI-C18:2 as Ld-preferring and non-raft probes, to avoid any confusions[s]
			Complementary to CTB				
NBD-DPPE (Hd)[t]	Variable (including the data obtained in supported bilayers and monolayers)	25 157 158	Lo-phase domain (clear images):	104	N.D.		Better not to use, to avoid any confusions[u]
			Complementary to LRB-DPPE (Hd)	159			
All SM analogs containing a large organic fluorophore in the acyl chain, except for SM-C6-NBD (Acyl)[v], SM-C12-NBD (Acyl)[v], and SM-C12-BodipyFL (Acyl)[v], examined thus far (including SM-TopFluor, SM-ATTO532, SM-ATTO647N, and SM-KK114 [all in the acyl chain])	Ld-phase domain (clear images) (for SM-TopFluor, SM-ATTO532, SM-ATTO647N, and SM-KK114, Lo/Ld < 0.3):	25 31 53	Ld-like domain (clear images) (for SM-TopFluor, SM-ATTO532, SM-ATTO647N, SM-KK114, and DPPC-C5-BodipyFL, Lo/Ld < 0.9):	30 53	N.D.	30	Better not to use, to avoid any confusions[w]
			Colocalization with TexasRed-DPPE (Hd), but no experiments showing complementary staining to raft-associated probes defined in the upper rows	30			

Cholesterol-TopFluor (Acyl)	Lo-phase domain (Lo/Ld = 4.0 ± 0.15)	53	Lo-like domain (Lo/Ld = 1.9 ± 0.17): Complementary to DiO, DiI or DiD	53	N.D.	A good candidate for raft, but TBD (DRM, GPI-anchored protein patches)
Naphthopyrene	Lo-phase domain (clear image): Complementary to LRB-DOPE	25	Lo-like domain (clear image): Complementary to LRB-DOPE	27	N.D.	A good candidate for raft, but TBD (DRM, GPI-anchored protein patches)

Note to Table 1:

[a]When the values of Lo/Ld ratios in GUVs and/or GPMVs for a molecule were found in the literature, they are given in the parenthesis. Even when the values of Lo/Ld ratios are not known for a molecule, the molecule is listed here if their partitioning is clear in the published images. In these cases, we state "clear image(s)", i.e., in such cases, the preferences given here are only qualitative, and the levels of preferences vary greatly. Despite these preferences, it is important to realize that these molecules additionally enter and/or interact with non-preferred domains extensively, undergoing very dynamic interactions with both domains extensively (see text).

Even when a molecule's Lo/Ld ratios in GUVs and GPMVs were not measured (indicated as "clear image"), since the Lo- and Ld-preferring molecules listed here exhibited very clear preferences in GUVs and GPMVs, the following are assumed. For Lo-preferring molecules, the ratios in these vesicles were assumed to be 1.5 or greater in GUVs and GPMVs (60% or more molecules partitioning into the Lo-phase and Lo-like domains). For Ld-preferring molecules, their GUVs' and GPMVs' Lo/Ld ratios were assumed to be 0.67 or smaller (60% or more molecules partitioning into the Ld-phase and Ld-like domains). These assumptions are consistent with the results summarized in Table 4.

[b]The results shown here include both the fluorescent imaging data after cold-Triton treatment of the fluorescently-labeled cells and the western blotting data after sucrose density-gradient fractionation of the cold-Triton-treated cells.

[c]Read this column after reading Section 9 "Working definition of raft domains" and Section 10 "Fluorescent lipid probes that might be useful for raft-domain research" in the main text. As described in Section 10, probe molecules that satisfy all the three conditions, (1) Lo (Ld)-phase-domain preference in GUVs, (2) Lo (Ld)-like-domain preference in GPMVs, and (3) DRM (non-DRM) preference, are classified into raft (non-raft) associated.

[d]LRB-DOPE; Lissamine rhodamine B sulfonyl-dioleoylphosphatidylethanolamine.

[e]DiI-C18:1, DiI-C18:2 [FastDiI], and DiO-C18:2 [FastDiO]; 1,1'-dioleoyl-3,3,3',3'-tetramethylindocarbo-cyanine methanesulfonate, 1,1'-dilinoleoyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, and 3,3'-dilinoleoyloxacarbocyanine perchlorate, respectively.

[f](Acyl) indicates that the fluorescent probe is attached to the acyl chain.

[g] *Monomeric* CTB (bound to a single molecule of GM1) partitioned more into Ld-like domains in GPMVs.[123]

[h] Native GM1 molecules highly partitioned into DRM. Furthermore, native GM1 molecules were almost completely recovered in the DRM of Brij98 at 37°C.[156]

[i] (Hd) indicates that the fluorophore is attached to the lipid headgroup.

[j] 488neg-SM(18:0) and 594neg-SM(18:0); ATTO488- and ATTO594-nonaethyleneglycol-sphingomyelin (18:0), respectively. In the molecules whose names contain "neg", a fluorophore is attached to the choline headgroup (without changing its positive charge) by way of nonaethylene glycol ($[-CH_2-CH_2-O-]_9$).

[k] This ratio is comparable to that found for native SM[C18:0].[79]

[l] 594neg-DSPC, 488neg-DSPC, and 594neg-DOPC; ATTO594-nonaethyleneglycol-L-α-distearoyl-PC, ATTO488-nonaethyleneglycol-L-α-distearoyl-PC, and ATTO594-nonaethyleneglycol-L-α-dioleoyl-PC, respectively.

[m] In the examinations using GPMVs, no experiments showing complementary staining with non-raft probes has been performed. However, since clear colocalization with 488neg-SM was observed, and since Lo/Ld ratio is high (4.5 ± 0.40), coupled with its high partitioning into Lo domains in GUVs and DRM, it is concluded that this probe 594neg-DSPC is very likely associated with raft domains. Therefore, in the following lines in this table, and in the text, 594neg-DSPC is treated as a raft-associated probe.

[n] N.D.; experiments not done.

[o] A related molecule 594neg-DSPC (a row above), in which the ATTO488 group was replaced by the ATTO594, has been judged to be a raft associated molecule. Since ATTO488 is more hydrophilic than ATTO594, and thus is expected to disturb the lipid interactions in the hydrophobic region less than ATTO594, it is very likely that 488neg-DSPC acts as a proper raft probe. Therefore, in the following lines in this table, and in the text, 488neg-DSPC is treated as such.

[p] TexasRed-DPPE (Hd), LRB-DPPE (Hd), and FITC-DPPE (Hd); TexasRed, Lissamine rhodamine B sulfonyl, and FITC linked to the amine group (headgroup) of DPPE.

[q] Clear colocalization of this probe (FITC-DPPE) with LRB-DOPE was found in GPMVs (see the first row in this table). This is probably due to the effect of the presence of the large hydrophobic dye in the headgroup, which would be incorporated in the hydrophobic part of the membrane, disturbing the order in Lo-like domains. This would block the entry of this probe (FITC-DPPE) into Lo-like domains in GPMVs. Since LRB-DPPE and TexasRed-DPPE include even larger and more hydrophobic fluorescent moieties, they are likely to partition more into Ld-like domains in GPMVs than FITC-DPPE. Furthermore, TexasRed-DPPE exhibited systematic changes in the staining of GUVs undergoing the miscibility transition.[98] Due to these reasons, it is very likely that TexasRed-DPPE (Hd), LRB-DPPE (Hd), and FITC-DPPE (Hd) behaves as proper non-raft probes. Therefore, in the following lines in this table and in the text, TexasRed-DPPE (Hd), LRB-DPPE (Hd), and FITC-DPPE (Hd) are treated as such.

Note that another DPPE-based probe containing a smaller fluorophore NBD, NBD-DPPE,

exhibited varied partitioning behaviors in GUVs (see the row further down and Note u).

Due to such complexities in the behaviors of these probes, we recommend rather using fluorescent probes containing dioleoyl chains as Ld-preferring and non-raft preferring probes.

Honigmann et al.[160] found that a fluorescent probe with somewhat similar structure, KK114-DSPE (Hd), partitions into the Ld domain, consistent with the result of TexasRed-DPPE (Hd), LRB-DPPE (Hd), and FITC-DPPE (Hd). Interestingly, when the flurophore KK114 was placed farther away from the membrane by inserting a polyethylene glycol chain (MW ~ 2 kD; PEG; this probe was called KK114-PEG-DSPE), KK114-PEG-DSPE preferentially partitioned into the Lo-phase domain, suggesting that KK114-PEG-DSPE could be a useful raft-associated probe. However, we did not list these molecules in this table because the experiments were performed in the mica-supported artificial bilayers, which might have affected the bilayer properties: the diffusion coefficient of KK114-PEG-DSPE is slower there by a factor of 3 ~ 5 compared with that in the Ld-phase domain in GUVs (see Table 2).

[r]diI-C16:0; 1,1'-dihexadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate.

[s]The partitioning behaviors of diI-C18:0, diI-C20:0, and diI-C22:0 in phase-separated GUVs are complex. Their partitioning is different between GUVs containing sphingomyelin or DSPC as saturated phospholipids (preferring Ld-phase domains or Lo-phase domains, respectively[25]). Due to such complexities in the behaviors of these probes, we recommend using diI-C18:1 and diI-C18:2 as Ld-preferring and non-raft preferring probe, rather than diI with saturated acyl chains.

[t]NBD-DPPE (Hd); N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-L-α-DPPE.

[u]NBD-DPPE (Hd) exhibited varied results in GUVs, supported bilayers, and monolayers, in contrast to the beheviors of related molecules such as TexasRed-DPPE (Hd), LRB-DPPE (Hd), and FITC-DPPE (Hd). Therefore we would not recommend using NBD-DPPE (Hd) as raft/non-raft specific molecules. See Table 4. See Note q.

[v]These fluorescent probes (SM-C6-NBD [Acyl], SM-C12-NBD [Acyl], and SM-C12-BodipyFL [Acyl]) exhibited non-consistent behaviors between GUVs and GPMVs, and in addition, the results obtained by different labs did not agree with each other (Table 4), and therefore we would not recommend using these molecules as raft/non-raft specific molecules.

[w]These SM probes do not behave like native SM molecules (Table 4) probably due to the disturbance by the attached fluorophores.[161] It is better not to use these probes because they might exhibit complicated behaviors and their names might mislead the readers.

Table 2. Diffusion coefficients of molecules in artificial membranes (GUVs and multilamellar vesicles [MLVs]; not limited to tertiary mixtures of lipids), GPMVs, PM spheres, and PMs.

Diffusant (Probe/molecule)	Membrane (lipid composition or cell line)	Temp (˚C)	*D*[a] in Lo-phase or Lo-like domains ($\mu m^2/s$)	*D* in Ld-phase or Ld-like domains ($\mu m^2/s$)	*D* in the homogeneous bulk domain (Ld or Lo) ($\mu m^2/s$)	Method	Ref
DiI-C_{18}[b]	GUV (SM/DOPC/Chol = 4.5:4.5:1)	~22	0.105 ± 0.031	4.9 ± 0.3	N.A.	FCS[c]	21
DiI-C_{18}	GUV (SM/DOPC/Chol = 2:2:1)	~22	0.255 ± 0.058	5.15 ± 0.15	N.A.	FCS	21
DiI-C_{18}	GUV (SM/DOPC/Chol = 1:1:1)	~22	0.795 ± 0.108	5.1 ± 0.4	N.A.	FCS	21
DiI-C_{18}	GUV (SM/DOPC/Chol = 5.3:1.3:3.3)	~22	0.8 ± 0.1	5.1 ± 0.9	N.A.	FCS	21
488neg-SM[d]	GUV (SM/DOPC/Chol = 1:1:1)	28.5	0.9 ± 0.1	5.5 ± 0.3	N.A.	FCS	31
594neg-SM[d]	GUV (SM/DOPC/Chol = 1:1:1)	28.5	0.9 ± 0.1	5.4 ± 0.2	N.A.	FCS	31
594neg-DSPC[e]	GUV (DSPC/DOPC/Chol = 1:1:1)	28.5	1.0 ± 0.1	5.5 ± 0.3	N.A.	FCS	31
594neg-DSPC	GUV (SM/DOPC/Chol = 1:1:1)	28.5	0.9 ± 0.1	4.9 ± 0.4	N.A.	FCS	31
594neg-DOPC[e]	GUV (SM/DOPC/Chol = 1:1:1)	28.5	0.9 ± 0.1	6.0 ± 0.2	N.A.	FCS	31
IL-4Rα[f] (EGFP linked; mostly located in the Ld-phase domain)	GUV (DOPC/SM/Chol =1:1:1)	22	Not measurable	8.2 ± 2.0	N.A.	FCS	85
Fast-DiO[g] (Ld-phase preferring probe)	GUV (DOPC/SM/Chol = 1:1:1)	22	Not measurable	11.7 ± 1.6	N.A.	FCS	85
PLAP[h] (rhodamine label)	GUV (SM/DOPC/Chol =1:1:1)	~22	0.7 ± 0.3	3.8 ± 0.3	N.A.	FCS	84
PLAP (rhodamine label)	GUV (DOPC)	~22	N.A.	N.A.	(Ld-phase) 5.0 ± 0.3	FCS	84
PLAP (rhodamine label)	GUV (DOPC)	~22	N.A.	N.A.	(Ld-phase) 6.3 ± 0.3	FCS	21
DPPC-d_{62}[i]	MLV (DPPC-d_{62} without Chol)	46	N.A.	N.A.	(Ld-phase) 12.1	PFG MAS NMR[j]	86
DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 9.5:0.5)	46	N.A. N.A.	N.A. N.A.	(Ld-phase) 12.0 14.3	PFG MAS NMR	86
DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 3:2)	46	N.A. N.A.	N.A. N.A.	(Lo-phase) 5.2 7.2	PFG MAS NMR	86

DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 1:1)	46	N.A. N.A.	N.A. N.A.	(Lo-phase) 4.3 5.2	PFG MAS NMR	86
DPPC-d_{62}	MLV (DPPC-d_{62} without Chol)	51	N.A.	N.A.	(Ld-phase) 15.1	PFG MAS NMR	86
DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 9.5:0.5)	51	N.A. N.A.	N.A. N.A.	(Ld-phase) 14.4 17.7	PFG MAS NMR	86
DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 3:2)	51	N.A. N.A.	N.A. N.A.	(Lo-phase) 6.3 9.7	PFG MAS NMR	86
DPPC-d_{62} Chol	MLV (DPPC-d_{62}/Chol = 1:1)	51	N.A. N.A.	N.A. N.A.	(Lo-phase) 5.4 7.2	PFG MAS NMR	86
Rhodamine 1-stearoyl 2-oleoyl-PE	GPMV (NIH 3T3 fibroblasts)	10	1.8	5.6	N.A.	FCS	29
CTB-Alexa488[k]	PM sphere (A431 cells) CTB-induced Lo- and Ld-like domains	37 or RT?	0.13[l] (+0.06, -0.11)	1.3[l] (+0.2, -0.4)	N.A.	FCS	28
VIP17/MAL-mRFP[m]	PM sphere (A431 cells) CTB-induced Lo- and Ld-like domains	37 or RT?	0.10[n] ± 0.03	1.27[n] ± 0.25	(no CTB addition; single domain) 1.34[n] ± 0.72	FCS	28
IL-4Rα (EGFP linked; mostly located in Ld)	PM (HEK293-derived cell line)	22	N.A.	N.A.	(mostly Ld) 0.38 ± 0.15	FCS	85
Fast-DiO (Ld-phase domain and Ld-like domain preferring probe)	PM (HEK293-derived cell line)	22	N.A.	N.A.	(mostly Ld) 1.3 ± 0.4	FCS	85
IL-4Rα (EGFP linked; mostly located in Ld-like domains)	GPMV (HEK293-derived cell line)	22	N.A.	N.A.	(mostly Ld) 1.5 ± 0.6	FCS	85
Fast-DiO (Ld-like domain preferring probe)	GPMV (HEK293-derived cell line)	22	N.A.	N.A.	(mostly Ld) 2.5 ± 0.5	FCS	85
Alexa fluor488-GltT (single cysteine mutant of the glutamate transporter GltT(Q412C))[o]	Large unilamellar vesicles (LUVs) DOPE/DOPG (3:1)	RT?	N.A.	N.A.	2.2 ±0.4 ~ 4.2 ± 0.2 (4.1-nm radius)[r]	FCS	162
Alexa fluor488-LacS (single cysteine mutants of the lactose transporter LacS (A535C))[p]	Large unilamellar vesicles (LUVs) DOPE/DOPG (3:1)	RT?	N.A.	N.A.	2.8 ± 1.2~ 5.0 ± 0.8 (3.3-nm radius)[r]	FCS	162
Alexa fluor488-LacY (lactose permease LacY C154G/S401C mutant)[q]	Large unilamellar vesicles (LUVs) DOPE/DOPG (3:1)	RT?	N.A.	N.A.	4.2 ± 0.6~ 4.6 ± 0.6 (2.1-nm radius)[r]	FCS	162

Note to Table 2:

[a] *D*; diffusion coefficient.

[b] DiI-C_{18}; 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate.

[c] FCS; fluorescence correlation spectroscopy.

[d]488neg-SM and 594neg-SM; ATTO488- and ATTO594-nonaethyleneglycol-sphingomyelin (18:0), respectively.

[e]594neg-DSPC and 594neg-DOPC; ATTO594-nonaethyleneglycol-L-α-DSPC and DOPC, respectively.

[f]IL-4Rα; single-pass TM protein, N-terminally His-tagged interleukin-4 receptor α chain comprising the amino acids 1-266 fused to a short-linker ADPPV and a C-terminal EGFP (540 amino acids in total; signal deficient due to truncation of the cytoplasmic domain), mostly located in the Ld-phase domain in GUVs and Ld-phase-like domain in GPMVs at 22˚C.

[g]Fast-DiO; 3,3′-dilinoleoyloxa-carbocyanine perchlorate (Ld-preferring probe).

[h]PLAP; human placental alkaline phosphatase.

[i]DPPC-d_{62}; L-α-dipalmitoyl-d_{62}-phosphatidylcholine.

[j]PFG MAS NMR; ^{1}H pulsed-field gradients magic-angle spinning NMR spectroscopy.

[k]CTB-Alexa488; CTB conjugated with Alexa488.

[l]These values are smaller than those of other molecules shown in this table by factors of 4 - 20. If no actin-based MSK exists in the PM spheres at all, the larger radius of CTB-GM1 pentamer complex will not explain such strong diffusion suppression of CTB-GM1 pentamer complex in PM spheres, following the theory of Saffman and Delbrück,[163] which was extensively proved by experiments (for a review, see Ref. 101 and 11), including the results shown in bottom three rows in this table (cf. Note r). This suggests that small amounts of the actin-based MSK is still attached to the cytoplasmic surface of PM spheres.

[m]VIP17/MAL-mRFP; vesicular integral membrane protein 17/myelin and lymphocyte protein (tetraspanning proteolipid) conjugated by mRFP.

[n]These values are smaller than those of other molecules shown in this table by factors of 4 - 20. The reason for the difference is unknown. However, as described in Note l, small amounts of the actin-based MSK might still be attached to the cytoplasmic surface of PM spheres and VIP17/MAL-mRFP might be in molecular complexes. When these two occur at the same time, diffusion would be slowed due to a phenomenon called "oligomerization-induced trapping" within a compartment generated by the actin-based membrane skeleton mesh.[164]

[o]From *Bacillus stearothermophilus*.

[p]From *Streptococcus thermophiles*.

[q]From *Escherichia coli*.

[r]The diffusion coefficients found in this work show a very weak dependence of the diffusion coefficients on the diffusant (protein) radius, and this dependence agreed well with the prediction made by Saffman and Delbrück.[163] Meanwhile, Gambin et al.[165] reported that the translational diffusion coefficient of the TM protein in the membrane was strongly radius-dependent, inversely proportional to their radius R (1/R model). However, the results by Gambin et al.[165] were obtained for synthetic model peptides reconstituted into surface-supported bilayers made of nonionic surfactants, and not native lipids such as phospholipids. Therefore, we tend to believe the results by Ramadurai et al.[162] 1and many others (for a review,

see Ref. 101 and 11) and the prediction made by Saffman and Delbrück.[163]

Table 3. Partitioning of proteins into raft/non-raft domains in the PM, expected from their partitioning into the Lo/Ld-like domains in GPMVs at ~10˚C (RBL-2H3 cells) and into DRM/non-DRM. The preferences in the partitioning in GPMVs was determined by complementary and/or colocalized distributions of fluorescent lipid probes with Lo/Ld preferences in GPMVs (Table 1). The "raft domain" in this table is that defined in Section 9.

Molecules	Preferences in GPMVs at lower tempera-tures[a]	Molecules examined for comple-mentary/ colocalized distributions in GPMVs	Ref	Parti-tioning in DRMs at ≤4˚C[b]	Ref	Preferences in GUVs	Ref	Expected associa-tion with raft/non-raft domains in the PM[c]
Transferrin receptor	Ld-like domain	Complementary to CTB[d] Colocalized with FastDiO and FastDiI	28 166	non-DRM	49 137 155 167	No direct GUV data available[e]		Very likely to be raft[e]
GPI-anchor (EGFP-GPI, YFP-GL-GPI[f], mYFP-GL-GPI[f])	Lo-like domain	Complementary to TexasRed-DPPE	104 109	DRM	43	Complicated[g]	84 168	Raft[h]
CD59 full length	Lo-like domain	Complementary to TexasRed-DPPE	109	DRM	43	Complicated[g]	84 168	Raft[h]
Thy-1 full length	Lo-like domain (Thy-1 crosslinked by mAb Ox 7)	Complementary to LRB-DOPE	27	DRM	137	Complicated[g]	84 158 168	Raft[h]
Anti GD1b mAb AA4 (forming dimers of acetylated GD1b gangliosides)	Lo-like domain (clear image)	Complementary to LRB-DOPE	104	N.D.		N.D.		TBD (GUV, DRM, and antibody-induced patches of GPI-anchored proteins)[i]
High-affinity IgE receptor[j]	Ld-like domain	Colocalized with LRB-DOPE	27	non-DRM	169-171	No direct GUV data available[e]		Very likely to be non-raft[e]
EGFP-Lck-anchor[k]	Varied	Co-stained with TexasRed-DPPE	109	DRM[l] (full length Lck)	172	N.D.		Probably complicated
EGFP-Fyn-anchor[j,k]	Ld-like domain	Colocalized with TexasRed-DPPE	109	DRM (full length Fyn)	155	N.D.		Probably complicated
EGFP-H-Ras anchor[j,m]	Ld-like domain	Colocalized with TexasRed-DPPE	109	DRM	173	N.D.		Probably complicated
H-Ras full length[j,m]	Ld-like domain	Colocalized with TexasRed-DPPE	109	Both GTP (GDP) form, more into non-DRM (DRM)	167, 173	N.D.		Probably complicated

Notes to Table 3

[a]The results included those performed in PM spheres at 37˚C as well as those observed in GPMVs at 10˚C.

[b]There have been attempts to prepare DRMs at 37˚C using different detergent (Brij 96[174]) and the DRMs of nano-meso scales.[48,175] However, since solubilizing the cells using 1% Triton X-100 at temperatures ≤4˚C is prevalent, we only list those using this protocol. The results shown here include both the fluorescent imaging data after cold Triton treatment of the cells and the western blotting data after sucrose density-gradient fractionation of the cold-Triton-treated cells.

[c]Section 9 "Working definition of raft domains" and Section 10 "Fluorescent lipid probes that might be useful for raft-domain research" in the main text.

[d]Note that this result was obtained in the PM sphere at 37˚C where phase separation was induced by the addition of CTB.

[e]Partitioning of transferrin receptor into Lo- and Ld-phase domains in phase-separated GUVs has not been examined. However, since interleukin receptor 4α chain (a single-pass TM protein) and bacteriorhodopsin (a 7-pass TM protein) partition into the Ld-phase domain in GUVs, it is likely that transferrin receptor partitions into the Ld-phase domain in GUVs.[84,85] Therefore, it is very likely that transferrin receptor is associated with non-raft domains.

[f]YFP-GL-GPI (and mYFP-GL-GPI) is an artificial GPI-anchored protein made from YFP (mYFP) containing a consensus N-glycosylation (GL) site fused to a GPI-attachment signal.

[g]In GPMVs, GPI-anchored proteins, such as EGFP-GPI-anchor, CD59, and Thy-1,[27,109] preferentially partition into Lo-like domains. Meanwhile, Kahya et al.[84] and Kahya and Schwille[168] showed that PLAP, a GPI-anchored protein, prefers Ld-phase domain over Lo-phase domain ~3:1 in GUVs at room temperature (perhaps ~22˚C). When PLAP was crosslinked by antibodies, the crosslinked PLAP did not show any preferences between Ld- and Lo-phase domains in GUVs (although PLAP apparently exists as constitutive dimers, which withstand even in SDS-PAGE[176]).

As touched upon in the main text, the lipid packing (order) evaluated by C-Laurdan was much higher in Lo-phase domains than that in Lo-like domains in GPMVs.[53,58] The high lipid order in Lo-phase domains in GUVs might drive PLAP (and perhaps other GPI-anchored proteins) away to partition more into Ld-phase domains.

Surprisingly, in supported lipid bilayers with equimolar mixture of SM (brain SM, which is predominantly 18:0), cholesterol, and DOPC, the majority of PLAP was found in the Lo-phase domain at room temperature, perhaps at ~22˚C.[176] Furthermore, in supported monolayers, another GPI-anchored protein, Thy-1, partitioned more into the Lo-phase domain (~2:1 over the Ld-phase domain;[158]). These variations in terms of GPI-anchored proteins' partitioning into Lo-phase domains in GUVs suggest that their partitioning into Lo-phase domains is determined by delicate balances of various molecular interactions.

[h]Despite the complicated behaviors of GPI-anchored proteins in artificial membranes (GUVs

and supported bilayers and monolayers) as described in the Note g, since all of the GPI-anchored proteins exhibited clear partitioning into the Lo-like domains in GPMVs and DRM, they were classified in the category of molecules that could associate with raft domains.

[i]The partitioning behaviors in GUV, DRM, and antibody-induced patches of GPI-anchored proteins (such as CD59) are to be determined.

[j]In older literature, TM proteins, hemagglutinin (HA), and linker for activation of T cells (LAT), and signaling proteins located on/in the PM cytoplasmic surface/leaflet, Fyn and H-Ras, were considered DRM-associated proteins. However, more recent results[109,177] suggest that more quantitative evaluation of DRM association in GPMVs would be necessary. Hemagglutinin partitioning in GPMVs vary greatly from a vesicle to another, exhibiting Lo, Ld, and no preferences at the ratios of 26, 58, and 16%.[109] The full length, palmitoylated LAT partitions into Lo-like domain more than the non-palmitoylated LAT, whereas the TM domain of LAT partitioned into the Lo and Ld domains almost equally in GPMVs.[166]

[k]Both the Lck-anchor and Fyn-anchor contain the sites for two palmitoyl chains and one myristoyl chain, but their behaviors in GPMVs are different.[109] The peptide sequences themselves and/or acyl chain modification efficiencies might affect the results.

[l]After Brij98 extraction at 37˚C, the full-length Lck partitioned into both DRM and non-DRM, but more in non-DRM.[156]

[m]The H-Ras anchor contains two palmitoyl chains and one farnesyl chain.

Table 4. Additional fluorescent lipid probes recommended for use as raft/non-raft-associated probes, based on more quantitative evaluations,, in various lipid categories. Unlike Tables 1 and 3, this table is based on more quantitative evaluations of the probe's partitioning into Lo-/Ld-phase domains in phase-separated GUVs (consisting of tertiary mixtures of saturated phospholipid (mostly Brain SM/DOPC/Chol = 1/1/1 [28.5°C][31] or 2/2/1 [~22°C][53]) and into complementary Lo-/Ld-like domains in GPMVs (RBL-2H3 cells) at 10°C (temperature unknown in Ref. 104), and into DRMs vs. intact-PM (T24 cells), as well as qualitative examination of colocalization within CD59 patches (T24 cells).

We propose here the following way of determining preferential association of these probes with "raft" or "non-raft" domains (shown in the second column from the right). However, this categorization is basically over-simplified, and one should always keep in mind that these probes partition into both "raft" or "non-raft" domains anyway, i.e., the preference is not all or none.

(1) If the Lo/Ld ratio in GPMV is 1.5 or greater, we refer to the data of GUV partitioning, DRM incorporation, and localization within antibody-induced patches of GPI-anchored protein. If two or more of the following three conditions are satisfied, the molecule is determined to be raft-domain-associated. 1) The GUV Lo/Ld ratio ≥ 1.5; 2) DRM/intact-PM intensity ratio ≥ 0.67; and 3) localization within antibody-induced patches of GPI-anchored protein (CD59) = +. If at least one of them is not satisfied, the probe molecule is defined as "Not useful" as a raft-associated probe or a non-raft-associated probe. Note that the molecules classified into the "Not useful" category might be useful for other objectives: for example, they might partition into both domains and could be useful for monitoring the dynamics of the molecules in both raft and non-raft domains.

(2) If the Lo/Ld ratio in GPMV is 0.67 or smaller, we refer to the data of GUV partitioning, DRM incorporation, and localization within antibody-induced patches of GPI-anchored protein. If one of the following three conditions is satisfied, the molecule is defined to be non-raft-associated. 1) The GUV Lo/Ld ratio ≤ 0.67; 2) DRM/intact-PM intensity ratio < 0.33; and 3) localization within antibody-induced patches of GPI-anchored protein (CD59) = ± or –. If none of the three conditions are satisfied (when the GPMV's Lo/Ld ratio is 0.9 or smaller), the probe

molecule is defined as "Not useful" as a raft-associated probe or a non-raft-associated probe (although such probes have not been found and are not listed in this Table).

(3) In the case where the Lo/Ld ratio in GPMVs is greater than 0.67 and smaller than 1.5 (corresponding to 40 and 60% of the total number of molecules partitioning into the Lo domain), we classify them into the category of "Not useful as a probe for differentiating Lo/Ld-like domains in GPMVs".

Here, we heavily depend on the Lo/Ld ratio in GPMVs prepared from RBL-2H3 cells. Since molecular compositions of the GPMVs would be different from cell-type to cell-type, the use of this Table requires due caution.

Probes	GUV Lo/Ld partition ratio (mean ± SE)	GPMV Lo/Ld partition ratio (mean ± SE)	DRM/intact PM intensity ratio (mean ± SE)	Localiza-tion in CD59 patches	Association with "Raft" or "Non-raft" domains	Ref
Fluorescent ganglioside analogs						
Fl-S9-GM3 (Hd)[a]	N.D.	8.8 ± 1.5	1.1 ± 0.05	+	Raft	30
TMR-S9-GM3 (Hd)[a]	N.D.	1.5 ± 0.08	1.0 ± 0.10	+	Raft (weak)	30
594-S9-GM3 (Hd)[a]	N.D.	4.0 ± 0.14	1.0 ± 0.05	+	Raft	30
488-S9-GM3 (Hd)[a]	N.D.	4.6 ± 0.58	1.1 ± 0.06	+	Raft	30
647N-S9-GM3 (Hd)[a,b]	N.D.	0.26 ± 0.02	0.066 ± 0.008	–	Non-raft Better not to use[b]	30
TMR-G6-GM3 (Hd)[a]	N.D.	1.7 ± 0.14	0.029 ± 0.006	–	Not useful	30
594-G6-GM3 (Hd)[a]	N.D.	1.7 ± 0.09	0.49 ± 0.04	±	Not useful	30
594-S9-GM2 (Hd)[a]	N.D.	4.1 ± 0.48	1.0 ± 0.05	+	Raft	30
594-GN6-GM2 (Hd)[a]	N.D.	4.4 ± 0.43	1.1 ± 0.07	+	Raft	30
594-termG6-GD1b (Hd)[a]	N.D.	4.2 ± 0.40	1.0 ± 0.04	+	Raft	30
Cy3-CTB (GM1 pentamer)	Lo phase (no quantification)	6.7 ± 0.49	1.2 ± 0.08	+	Raft	21,30,178
TMR-S9-GM1 (Hd)[a]	N.D.	1.9 ± 0.25	1.0 ± 0.05	+	Raft (weak)	30
594-S9-GM1 (Hd)[a]	N.D.	3.3 ± 0.11	1.1 ± 0.08	+	Raft	30
488-S9-GM1 (Hd)[a]	N.D.	3.0 ± 0.23	1.1 ± 0.06	+	Raft	30
594-termG6-GM1 (Hd)[a]	N.D.	4.7 ± 0.35	1.0 ± 0.05	+	Raft	30
Alexa568-GM1 (Hd)	Lo~No Pref[c]	N.D.	N.D.	N.D.	N.D.	179
647N-GM1 (Hd)[d]	0.09 ± 0.01	0.35 ± 0.05	N.D.	N.D.	Non-raft Better not to use[b]	53
GM1-647N (Acyl)[d]	0.06 ± 0.02	0.12 ± 0.01	N.D.	N.D.	Non-raft Better not to use[b]	53
GM1-C5-BodipyFL (Acyl)[e]	0.33 ± 0.09	1.9 ± 0.17	N.D.	N.D.	Not useful[e]	53
GM1-C5-BodipyFL (Acyl)[e]	N.D.	0.62 ± 0.05	0.042 ± 0.009	–	Not useful[e]	30
GM1-C5-BodipyFL (Acyl)[e]	N.D.	Varied[b]	N.D.	N.D.	Not useful[e]	104
GM1-C6-NBD (Acyl)	0.30 ± 0.10	2.0 ± 0.18	N.D.	N.D.	Not useful	53
Fluorescent sphingomyelin (SM) analogs						
488neg-SM(18:0) (Hd)[f]	4.1 ± 0.15	4.8 ± 0.50	1.1 ± 0.06	N.D.	Raft	31
594neg-SM(18:0) (Hd)[f]	3.9 ± 0.15	4.7 ± 0.50	1.1 ± 0.07	N.D.	Raft	31
SM-TopFluor (Acyl)	0.27 ± 0.05	0.49 ± 0.09	N.D.	N.D.	Non-raft	53
SM-C12-BodipyFL (Acyl)	0.45 ± 0.07	1.9 ± 0.17	N.D.	N.D.	Not useful	53
SM-C12-NBD (Acyl)[g]	0.05 ± 0.02	0.53 ± 0.08	N.D.	N.D.	Not useful[d]	53
SM-C12-NBD (Acyl)[g] (also Ceramide-C12-NBD)	N.D.	~1[c]	N.D.	N.D.	Not useful[d]	104

SM-C6-NBD (Acyl)[h]	0.15 ± 0.03	0.85 ± 0.07	N.D.	N.D.	Not useful	53
SM-C6-NBD (Acyl)[h]	N.D.	Largely Lo	Intermediate	N.D.	Not useful[e]	104
SM-ATTO532 (Acyl)[d]	0.18 ± 0.04	0.88 ± 0.02	N.D.	N.D.	Not useful	53
ATTO532-SM (Hd)[d]	0.19 ± 0.05	0.61 ± 0.03	N.D.	N.D.	Non-raft Better not to use[b]	53
SM-ATTO647N (Acyl)[d]	0.04 ± 0.01	0.22 ± 0.06	N.D.	N.D.	Non-raft Better not to use[b]	53
ATTO647N-SM (Hd)[d]	0.03 ± 0.01	0.18 ± 0.07	N.D.	N.D.	Non-raft Better not to use[b]	53
SM- K114 (Acyl)[d]	0.02 ± 0.01	0.04 ± 0.02	N.D.	N.D.	Non-raft Better not to use[b]	53
K114-SM (Hd)[d]	0.05 ± 0.02	0.14 ± 0.01	N.D.	N.D.	Non-raft Better not to use[b]	53
Fluorescent PC analogs						
594neg-DSPC (Hd)[f]	4.3 ± 0.24	4.5 ± 0.40	1.1 ± 0.09	N.D.	Raft	31
594neg-DOPC (Hd)[f]	0.14 ± 0.01	0.20 ± 0.02	0.039 ± 0.003	N.D.	Non-raft	31
DPPC-C5-BodipyFL (Acyl)	Ld-phase	0.23 ± 0.02	non-DRM	N.D.	Non-raft	30,31
Fluorescent cholesterol analogs						
Chol-TopFluor (Acyl)[i]	4.0 ± 0.15	1.9 ± 0.17	N.D.	N.D.	N.D.	53
Chol-Bodipy-TMR (Acyl)[i]	0.69 ± 0.02	3.2 ± 0.17	N.D.	N.D.	Not useful	53

Note to Table 4:

[a]The chemical structures of these fluorescent ganglioside analogs are shown in Fig. 4.

Fluorescent modification of the sialyl group in GM1 has been done quite extensively, for example, by Spiegel et al.,[180,181] Goins et al.,[182] Marushchak et al.,[183] Mikhalyov et al.,[184] and Sachl et al.[185] However, these probes have not been characterized as extensively as the probes listed in this table, in terms of their use for raft-domain studies.

[b]These ganglioside and SM probes do not behave like native ganglioside and SM molecules, respectively, probably due to the disturbance by the attached fluorophores.[161] It is better not to use these probes as raft/non-raft specific probes because they might exhibit complicated behaviors, and in addition their names might mislead the readers.

[c]When the GUVs consisting of DPPC/DOPC/Chol (30/35/35 by mols) were observed at room temperature, no preferential partitioning was observed. However, when the GUVs consisting of DPPC/DOPC/Chol (40/40/20 by mols) were observed at room temperature, Lo-phase domain partitioning was observed in 60% of the GUVs used, whereas no preferential partitioning was found in the remaining 40%.

[d]In the literature, these probes have sometimes been used as raft-associated probes. However, it is clear that these probes are associated with Ld-phase domains in GUVs and Lo-like domains in GPMVs, and, based on the criteria, they are classified into the non-raft-specific category. These results clearly indicate that the previous observations made by using these probes as raft markers must be re-interpreted; the events observed were very likely to take place in the bulk non-raft domain.

We noted that virtually all of the lipid probes that employed ATTO647N (both Hd and Acyl linked) preferred Ld-phase domains in GUVs and Ld-like domains in GPMVs, suggesting that the hydrophobic bulky fluorophore might be inserted into the hydrophobic environment around the probe molecule, causing the exclusion of the ATTO647N-linked lipids away from the Lo-phase/Lo-like domains. Therefore, it is likely that all the results obtained with ATTO647N-linked lipids should be re-interpreted as the events that occurred in the non-raft domain.

[e]The same probe, GM1-C5-BodipyFL (Acyl), was used in three independent examinations in different labs. Different results were obtained for the partitioning in GPMVs (perhaps Sengupta et al.[104] might have employed temperatures somewhat higher than 10°C, as they reported varied results for individual GPMVs), suggesting the lack of robustness of the results. Therefore, this probe was classified into the category of "Not useful". The results obtained by Sezgin et al.[53] by themselves indicate the same classification.

[f]The chemical structures of these fluorescent sphingomyelin and PC analogs are shown in Fig. 2.

[g]The same probe, SM-C12-NBD (Acyl), was used in two independent examinations in different labs. Different results were obtained for the partitioning in GPMVs, suggesting the lack of robustness of the results. Therefore, this probe was classified into the category of "Not useful". A probe with related structure, Ceramide-C12-NBD, did not exhibit any clear partitioning in GPMVs,[104] and therefore was classified into the category of "Not useful".

[h]The same probe, SM-C6-NBD (Acyl), was used in two independent examinations in different labs. Different results were obtained for the partitioning in GPMVs. According to Sengupta et al.,[104] SM-C6-NBD (Acyl) partitioned into the Lo-like domain in 80–90% of the GPMVs and partitioned more evenly between Lo-like and Ld-like domains in 10-20% of the GPMVs. These results are quite different from the results reported by Sezgin et al.[53] (partitioning more in Ld-like domain than in Lo-like domain), suggesting the lack of robustness of the results. Therefore, this probe was classified into the category of "Not useful".

[i]Satisfactory fluorescent cholesterol analogs have not been produced yet. None of the existing cholesterol analog probes induced higher packing of saturated phospholipid acyl chains, which native cholesterol induces. According to Scheid et al.[186], only a spin-labeled cholesterol with the doxyl group placed at the end of the acyl chain and a fluorescent probe cholestatrienol could mimic cholesterol's ordering effect to a certain degree.

Depending on the properties of the conjugated fluorescent/paramagnetic groups and/or their binding sites in cholesterol or chemical modifications of cholesterol to make it fluorescent, cholesterol analogs may adopt an "up-side-down" orientation in the membrane, undergo flip-flop faster than cholesterol, and/or fluctuate between "upright" and up-side-down orientation by rotational motions about the short axis not typical for native cholesterol. Such analogs are not able to induce condensation of saturated phospholipids (enhancing order) as much as cholesterol.[186]

Scheid et al.[186] showed that dehydroergosterol, which has been used quite extensively,[76,77] has an up-right orientation similar to that of cholesterol although it failed to induce lipid packing. They argued that, nevertheless, dehydroergosterol could be used at lower concentrations (up

to 1 mol%). Under these conditions, it might not perturb Lo-phase/Ld-like domain organization.

The problems of using cholestatrienol (absorption/emission maxima ~ 325/376 nm) and dehydroergosterol (absorption/emission maxima ~ 327/393 nm) are their poor signal to noise ratios under the fluorescence microscope. Their excitation wavelengths are in the UV range and therefore their observations require an UV fluorescence microscope or a two-photon excitation microscope. Even if these microscopes are employed, high quality images are difficult to obtain. In addition, they are quite susceptible to photobleaching and oxidation.

22-NBD-cholesterol (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5- cholen-3β-ol) and 25-NBD-cholesterol (25-{N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)- methyl]amino}-27-norcholesterol) were found to partition into Ld-phase domains in GUVs.[53,104] Furthermore, their NBD moiety is preferentially localized in the polar interface (the analog molecule is placed in the membrane upside down) and/or (ii) the analogs have very high transbilayer mobility. Neither 22- nor 25-NBD-cholesterol could induce higher packing of phospholipid alkyl chains, which native cholesterol induces. These results indicate that the use of NBD-cholesterol will not be useful for raft-domain research. Although unclear, similar problem might be occurring for Chol-TopFluor (Acyl) and Chol-Bodipy-TMR (Acyl).

Baumgart et al.[25] reported that polycyclic aromatic hydrocarbons, such as naphthopyrene and terrylene, because of their planar rigid shapes, partition into Lo-phase domains in GUVs and orient their long axes parallel to the bilayer normal, but that in a single Ld-phase domain, they are not oriented in any direction (their orientations are randomized).

Recently, Liu et al.[75] developed a collection of tunable orthogonal cholesterol sensors that seamlessly cover a wide dynamic range of cholesterol concentration down to <1 mol%. These sensors are genetically modified from the D4 domain of perfringolysin O, which is known to have high specificity for cholesterol. Such sensors would be extremely useful for the future raft research.[187]

References

1. Simons K, van Meer G. Lipid sorting in epithelial cells. *Biochemistry*. 1988;27(17):6197-6202. doi:10.1021/bi00417a001.

2. Simons K, Ikonen E. Functional rafts in cell membranes. *Nature*. 1997;387(6633):569-572. doi:10.1038/42408.

3. Subczynski WK, Kusumi A. Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. *Biochim Biophys Acta*. 2003;1610(2):231-243. doi:10.1016/S0005-2736(03)00021-X.

4. Kusumi A, Koyama-Honda I, Suzuki K. Molecular Dynamics and Interactions for Creation of Stimulation-Induced Stabilized Rafts from Small Unstable Steady-State Rafts. *Traffic*. 2004;5(4):213-230. doi:10.1111/j.1600-0854.2004.0178.x.

5. Kirkham M, Parton RG. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta*. 2005;1745(3):273-286. doi:10.1016/j.bbamcr.2005.06.002.

6. Nicolau DV, Burrage K, Parton RG, Hancock JF. Identifying optimal lipid raft characteristics required to promote nanoscale protein-protein interactions on the plasma membrane. *Mol Cell Biol*. 2006;26(1):313-323. doi:10.1128/MCB.26.1.313-323.2006.

7. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. *Science*. 2010;327(5961):46-50. doi:10.1126/science.1174621.

8. Simons K, Gerl MJ. Revitalizing membrane rafts: new tools and insights. *Nat Rev Mol Cell Biol*. 2010;11(10):688-699. doi:10.1038/nrm2977.

9. Simons K, Sampaio JL. Membrane organization and lipid rafts. *Cold Spring Harb Perspect Biol*. 2011;3(10):a004697-a004697. doi:10.1101/cshperspect.a004697.

10. Kusumi A, Suzuki KGN, Kasai RS, Ritchie K, Fujiwara TK. Hierarchical mesoscale domain organization of the plasma membrane. *Trends Biochem Sci*. 2011;36(11):604-615. doi:10.1016/j.tibs.2011.08.001.

11. Kusumi A, Fujiwara TK, Chadda R, et al. Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annu Rev Cell Dev Biol*. 2012;28(1):215-250. doi:10.1146/annurev-cellbio-100809-151736.

12. Kusumi A, Fujiwara TK, Morone N, et al. Membrane mechanisms for signal

transduction: The coupling of the meso-scale raft domains to membrane-skeleton-induced compartments and dynamic protein complexes. *Semin Cell Dev Biol*. 2012;23(2):126-144. doi:10.1016/j.semcdb.2012.01.018.

13. Levental I, Veatch S. The Continuing Mystery of Lipid Rafts. *J Mol Biol*. 2016;428(24 Pt A):4749-4764. doi:10.1016/j.jmb.2016.08.022.

14. Lorent JH, Diaz-Rohrer B, Lin X, et al. Structural determinants and functional consequences of protein affinity for membrane rafts. *Nat Comms*. 2017;8(1):1219. doi:10.1038/s41467-017-01328-3.

15. Kusumi A, Suzuki K. Toward understanding the dynamics of membrane-raft-based molecular interactions. *Biochim Biophys Acta*. 2005;1746(3):234-251. doi:10.1016/j.bbamcr.2005.10.001.

16. Sevcsik E, Schütz GJ. With or without rafts? Alternative views on cell membranes. 2016;38(2):129-139. doi:10.1002/bies.201500150.

17. Shankar J, Boscher C, Nabi IR. Caveolin-1, galectin-3 and lipid raft domains in cancer cell signalling. *Essays Biochem*. 2015;57:189-201. doi:10.1042/bse0570189.

18. Parton RG, Tillu VA, Collins BM. Caveolae. *Curr Biol*. 2018;28(8):R402-R405. doi:10.1016/j.cub.2017.11.075.

19. Parton RG. Caveolae: Structure, Function, and Relationship to Disease. *Annu Rev Cell Dev Biol*. 2018;34:111-136. doi:10.1146/annurev-cellbio-100617-062737.

20. Almeida PF, Vaz WL, Thompson TE. Percolation and diffusion in three-component lipid bilayers: effect of cholesterol on an equimolar mixture of two phosphatidylcholines. *Biophys J*. 1993;64(2):399-412. doi:10.1016/S0006-3495(93)81381-X.

21. Kahya N, Scherfeld D, Bacia K, Poolman B, Schwille P. Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy. *J Biol Chem*. 2003;278(30):28109-28115. doi:10.1074/jbc.M302969200.

22. Veatch SL, Keller SL. Organization in lipid membranes containing cholesterol. *Phys Rev Lett*. 2002;89(26):268101. doi:10.1103/PhysRevLett.89.268101.

23. Veatch SL, Keller SL. A closer look at the canonical "Raft Mixture" in model membrane studies. *Biophys J*. 2003;84(1):725-726. doi:10.1016/S0006-3495(03)74891-7.

24. Veatch SL, Keller SL. Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Phys Rev Lett*. 2005;94(14):148101. doi:10.1103/PhysRevLett.94.148101.

25. Baumgart T, Hunt G, Farkas ER, Webb WW, Feigenson GW. Fluorescence probe partitioning between Lo/Ld phases in lipid membranes. *Biochim Biophys Acta*.

2007;1768(9):2182-2194. doi:10.1016/j.bbamem.2007.05.012.

26. Bezlyepkina N, Gracià RS, Shchelokovskyy P, Lipowsky R, Dimova R. Phase Diagram and Tie-Line Determination for the Ternary Mixture DOPC/eSM/Cholesterol. *Biophys J*. 2013;104(7):1456-1464. doi:10.1016/j.bpj.2013.02.024.

27. Baumgart T, Hammond AT, Sengupta P, et al. Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc Natl Acad Sci USA*. 2007;104(9):3165-3170. doi:10.1073/pnas.0611357104.

28. Lingwood D, Ries J, Schwille P, Simons K. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc Natl Acad Sci USA*. 2008;105(29):10005-10010. doi:10.1073/pnas.0804374105.

29. Levental I, Byfield FJ, Chowdhury P, Gai F, Baumgart T, Janmey PA. Cholesterol-dependent phase separation in cell-derived giant plasma-membrane vesicles. *Biochem J*. 2009;424(2):163-167. doi:10.1042/BJ20091283.

30. Komura N, Suzuki KGN, Ando H, et al. Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat Chem Biol*. 2016;12(6):402-410. doi:10.1038/nchembio.2059.

31. Kinoshita M, Suzuki KGN, Matsumori N, et al. Raft-based sphingomyelin interactions revealed by new fluorescent sphingomyelin analogs. *J Cell Biol*. 2017;216(4):1183-1204. doi:10.1083/jcb.201607086.

32. Pierce SK, Liu W. The tipping points in the initiation of B cell signalling: how small changes make big differences. *Nat Rev Immunol*. 2010;10(11):767-777. doi:10.1038/nri2853.

33. Sengupta P, Jovanovic-Talisman T, Skoko D, Renz M, Veatch SL, Lippincott-Schwartz J. Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. *Nat Methods*. 2011;8(11):969-975. doi:10.1038/nmeth.1704.

34. Shelby SA, Holowka D, Baird B, Veatch SL. Distinct Stages of Stimulated FcεRI Receptor Clustering and Immobilization Are Identified through Superresolution Imaging. *Biophys J*. 2013;105(10):2343-2354. doi:10.1016/j.bpj.2013.09.049.

35. Castro BM, Torreno-Piña JA, van Zanten TS, Gracia-Parajo MF. Biochemical and imaging methods to study receptor membrane organization and association with lipid rafts. *Methods Cell Biol*. 2013;117:105-122. doi:10.1016/B978-0-12-408143-7.00006-2.

36. Kusumi A, Tsunoyama TA, Hirosawa KM, Kasai RS, Fujiwara TK. Tracking single molecules at work in living cells. *Nat Chem Biol*. 2014;10(7):524-532. doi:10.1038/nchembio.1558.

37. Eggeling C. Super-resolution optical microscopy of lipid plasma membrane dynamics. *Essays Biochem*. 2015;57:69-80. doi:10.1042/bse0570069.

38. Shelby SA, Veatch SL, Holowka DA, Baird BA. Functional nanoscale coupling of Lyn kinase with IgE-FcεRI is restricted by the actin cytoskeleton in early antigen-stimulated signaling. Lippincott-Schwartz J, ed. *Mol Biol Cell*. 2016;27(22):3645-3658. doi:10.1091/mbc.e16-06-0425.

39. Honigmann A, Pralle A. Compartmentalization of the Cell Membrane. *J Mol Biol*. 2016;428(24 Pt A):4739-4748. doi:10.1016/j.jmb.2016.09.022.

40. Goyette J, Gaus K. Mechanisms of protein nanoscale clustering. *Curr Opin Cell Biol*. 2017;44:86-92. doi:10.1016/j.ceb.2016.09.004.

41. Surma MA, Klose C, Simons K. Lipid-dependent protein sorting at the trans-Golgi network. *Biochim Biophys Acta*. 2012;1821(8):1059-1067. doi:10.1016/j.bbalip.2011.12.008.

42. Zurzolo C, Simons K. Glycosylphosphatidylinositol-anchored proteins: Membrane organization and transport. *Biochim Biophys Acta*. 2016;1858(4):632-639. doi:10.1016/j.bbamem.2015.12.018.

43. Suzuki KGN, Kasai RS, Hirosawa KM, et al. Transient GPI-anchored protein homodimers are units for raft organization and function. *Nat Chem Biol*. 2012;8(9):774-783. doi:10.1038/nchembio.1028.

44. Suzuki KGN, Ando H, Komura N, Fujiwara TK, Kiso M, Kusumi A. Development of new ganglioside probes and unraveling of raft domain structure by single-molecule imaging. *Biochim Biophys Acta*. 2017;1861(10):2494-2506. doi:10.1016/j.bbagen.2017.07.012.

45. Suzuki KGN, Ando H, Komura N, et al. Revealing the Raft Domain Organization in the Plasma Membrane by Single-Molecule Imaging of Fluorescent Ganglioside Analogs. *Meth Enzymol*. 2018;598:267-282. doi:10.1016/bs.mie.2017.06.038.

46. Heerklotz H. Triton promotes domain formation in lipid raft mixtures. *Biophys J*. 2002;83(5):2693-2701. doi:10.1016/S0006-3495(02)75278-8.

47. Brown DA. Analysis of raft affinity of membrane proteins by detergent-insolubility. *Methods Mol Biol*. 2007;398(Chapter 2):9-20. doi:10.1007/978-1-59745-513-8_2.

48. Morris RJ, Jen A, Warley A. Isolation of nano-meso scale detergent resistant membrane that has properties expected of lipid 'rafts'. *J Neurochem*. 2011;116(5):671-677. doi:10.1111/j.1471-4159.2010.07076.x.

49. Brown DA. Preparation of detergent-resistant membranes (DRMs) from cultured

mammalian cells. *Methods Mol Biol*. 2015;1232:55-64. doi:10.1007/978-1-4939-1752-5_5.

50. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell*. 1992;68(3):533-544. doi:10.1016/0092-8674(92)90189-J.

51. Ahmed SN, Brown DA, London E. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry*. 1997;36(36):10944-10953. doi:10.1021/bi971167g.

52. Fastenberg ME, Shogomori H, Xu X, Brown DA, London E. Exclusion of a transmembrane-type peptide from ordered-lipid domains (rafts) detected by fluorescence quenching: extension of quenching analysis to account for the effects of domain size and domain boundaries. *Biochemistry*. 2003;42(42):12376-12390. doi:10.1021/bi034718d.

53. Sezgin E, Levental I, Grzybek M, et al. Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim Biophys Acta*. 2012;1818(7):1777-1784. doi:10.1016/j.bbamem.2012.03.007.

54. Golfetto O, Hinde E, Gratton E. Laurdan fluorescence lifetime discriminates cholesterol content from changes in fluidity in living cell membranes. *Biophys J*. 2013;104(6):1238-1247. doi:10.1016/j.bpj.2012.12.057.

55. Klymchenko AS, Kreder R. Fluorescent probes for lipid rafts: from model membranes to living cells. *Chem Biol*. 2014;21(1):97-113. doi:10.1016/j.chembiol.2013.11.009.

56. Sezgin E, Levental I, Mayor S, Eggeling C. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat Rev Mol Cell Biol*. 2017;18(6):361-374. doi:10.1038/nrm.2017.16.

57. Ma Y, Benda A, Kwiatek J, Owen DM, Gaus K. Time-Resolved Laurdan Fluorescence Reveals Insights into Membrane Viscosity and Hydration Levels. *Biophys J*. 2018;115(8):1498-1508. doi:10.1016/j.bpj.2018.08.041.

58. Kaiser H-J, Lingwood D, Levental I, et al. Order of lipid phases in model and plasma membranes. *Proc Natl Acad Sci USA*. 2009;106(39):16645-16650. doi:10.1073/pnas.0908987106.

59. Morris RJ. Thy-1, a Pathfinder Protein for the Post-genomic Era. *Front Cell Dev Biol*. 2018;6:173. doi:10.3389/fcell.2018.00173.

60. Subczynski WK, Hopwood LE, Hyde JS. Is the mammalian cell plasma membrane a barrier to oxygen transport? *J Gen Physiol*. 1992;100(1):69-87.

doi:10.1085/jgp.100.1.69.

61. Green JM, Zheleznyak A, Chung J, et al. Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. *J Cell Biol*. 1999;146(3):673-682. doi:10.1083/jcb.146.3.673.

62. Surviladze Z, Dráberová L, Kovárová M, Boubelík M, Dráber P. Differential sensitivity to acute cholesterol lowering of activation mediated via the high-affinity IgE receptor and Thy-1 glycoprotein. *Eur J Immunol*. 2001;31(1):1-10. doi:10.1002/1521-4141(200101)31:1<1::AID-IMMU1>3.0.CO;2-W.

63. Suzuki KGN, Fujiwara TK, Sanematsu F, Iino R, Edidin M, Kusumi A. GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J Cell Biol*. 2007;177(4):717-730. doi:10.1083/jcb.200609174.

64. Sheets ED, Holowka D, Baird B. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J Cell Biol*. 1999;145(4):877-887. doi:10.1083/jcb.145.4.877.

65. Monastyrskaya K, Hostettler A, Buergi S, Draeger A. The NK1 receptor localizes to the plasma membrane microdomains, and its activation is dependent on lipid raft integrity. *J Biol Chem*. 2005;280(8):7135-7146. doi:10.1074/jbc.M405806200.

66. Vial C, Evans RJ. Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. *J Biol Chem*. 2005;280(35):30705-30711. doi:10.1074/jbc.M504256200.

67. Hunter I, Nixon GF. Spatial compartmentalization of tumor necrosis factor (TNF) receptor 1-dependent signaling pathways in human airway smooth muscle cells. Lipid rafts are essential for TNF-alpha-mediated activation of RhoA but dispensable for the activation of the NF-kappaB and MAPK pathways. *J Biol Chem*. 2006;281(45):34705-34715. doi:10.1074/jbc.M605738200.

68. Suzuki KGN, Fujiwara TK, Edidin M, Kusumi A. Dynamic recruitment of phospholipase C gamma at transiently immobilized GPI-anchored receptor clusters induces IP3-Ca2+ signaling: single-molecule tracking study 2. *J Cell Biol*. 2007;177(4):731-742. doi:10.1083/jcb.200609175.

69. Xu S, Huo J, Gunawan M, Su I-H, Lam K-P. Activated dectin-1 localizes to lipid raft microdomains for signaling and activation of phagocytosis and cytokine production in dendritic cells. *J Biol Chem*. 2009;284(33):22005-22011. doi:10.1074/jbc.M109.009076.

70. Seveau S, Bierne H, Giroux S, Prévost M-C, Cossart P. Role of lipid rafts in E-cadherin-

- and HGF-R/Met--mediated entry of Listeria monocytogenes into host cells. *J Cell Biol*. 2004;166(5):743-753. doi:10.1083/jcb.200406078.

71. Abrami L, Liu S, Cosson P, Leppla SH, van der Goot FG. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J Cell Biol*. 2003;160(3):321-328. doi:10.1083/jcb.200211018.

72. Kwik J, Boyle S, Fooksman D, Margolis L, Sheetz MP, Edidin M. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci USA*. 2003;100(24):13964-13969. doi:10.1073/pnas.2336102100.

73. Coskun Ü, Simons K. Membrane rafting: from apical sorting to phase segregation. *FEBS Lett*. 2010;584(9):1685-1693. doi:10.1016/j.febslet.2009.12.043.

74. Yang B, Oo TN, Rizzo V. Lipid rafts mediate H2O2 prosurvival effects in cultured endothelial cells. *FASEB J*. 2006;20(9):1501-1503. doi:10.1096/fj.05-5359fje.

75. Liu S-L, Sheng R, Jung JH, et al. Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. *Nat Chem Biol*. 2017;13(3):268-274. doi:10.1038/nchembio.2268.

76. Hao M, Mukherjee S, Maxfield FR. Cholesterol depletion induces large scale domain segregation in living cell membranes. *Proc Natl Acad Sci USA*. 2001;98(23):13072-13077. doi:10.1073/pnas.231377398.

77. Maxfield FR, Wüstner D. Analysis of cholesterol trafficking with fluorescent probes. *Methods Cell Biol*. 2012;108:367-393. doi:10.1016/B978-0-12-386487-1.00017-1.

78. Swamy MJ, Ciani L, Ge M, et al. Coexisting Domains in the Plasma Membranes of Live Cells Characterized by Spin-Label ESR Spectroscopy. *Biophys J*. 2005;90(12):4452-4465. doi:10.1529/biophysj.105.070839.

79. Yasuda T, Tsuchikawa H, Murata M, Matsumori N. Deuterium NMR of raft model membranes reveals domain-specific order profiles and compositional distribution. *Biophys J*. 2015;108(10):2502-2506. doi:10.1016/j.bpj.2015.04.008.

80. Marsh D. *Handbook of Lipid Bilayers*. 0 ed. CRC Press; 2013. doi:10.1201/b11712.

81. Veatch SL, Soubias O, Keller SL, Gawrisch K. Critical fluctuations in domain-forming lipid mixtures. *Proc Natl Acad Sci USA*. 2007;104(45):17650-17655. doi:10.1073/pnas.0703513104.

82. Heberle FA, Wu J, Goh SL, Petruzielo RS, Feigenson GW. Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains. *Biophys J*. 2010;99(10):3309-3318. doi:10.1016/j.bpj.2010.09.064.

83. Wu H-M, Lin Y-H, Yen T-C, Hsieh C-L. Nanoscopic substructures of raft-mimetic liquid-ordered membrane domains revealed by high-speed single-particle tracking. *Sci Rep*. 2016;6(1):20542. doi:10.1038/srep20542.

84. Kahya N, Brown DA, Schwille P. Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles. *Biochemistry*. 2005;44(20):7479-7489. doi:10.1021/bi047429d.

85. Worch R, Petrášek Z, Schwille P, Weidemann T. Diffusion of Single-Pass Transmembrane Receptors: From the Plasma Membrane into Giant Liposomes. *J Membr Biol*. 2016;279:37951–37914. doi:10.1007/s00232-016-9936-8.

86. Scheidt HA, Huster D, Gawrisch K. Diffusion of Cholesterol and Its Precursors in Lipid Membranes Studied by1H Pulsed Field Gradient Magic Angle Spinning NMR. *Biophys J*. 2004;89(4):2504-2512. doi:10.1529/biophysj.105.062018.

87. Bagatolli LA, Parasassi T, Fidelio GD, Gratton E. A model for the interaction of 6-lauroyl-2-(N,N-dimethylamino)naphthalene with lipid environments: implications for spectral properties. *Photochem Photobiol*. 1999;70(4):557-564.

88. Gaus K, Zech T, Harder T. Visualizing membrane microdomains by Laurdan 2-photon microscopy. *Mol Membr Biol*. 2006;23(1):41-48. doi:10.1080/09687860500466857.

89. Kim HM, Choo H-J, Jung S-Y, et al. A two-photon fluorescent probe for lipid raft imaging: C-laurdan. *ChemBioChem*. 2007;8(5):553-559. doi:10.1002/cbic.200700003.

90. Radhakrishnan A, McConnell H. Condensed complexes in vesicles containing cholesterol and phospholipids. *Proc Natl Acad Sci USA*. 2005;102(36):12662-12666. doi:10.1073/pnas.0506043102.

91. Rubenstein JL, Owicki JC, McConnell HM. Dynamic properties of binary mixtures of phosphatidylcholines and cholesterol. *Biochemistry*. 1980;19(3):569-573. doi:10.1021/bi00544a027.

92. Recktenwald DJ, McConnell HM. Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry*. 1981;20(15):4505-4510. doi:10.1021/bi00518a042.

93. Vist MR, Davis JH. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry*. 1990;29(2):451-464. doi:10.1021/bi00454a021.

94. Subczynski WK, Antholine WE, Hyde JS, Kusumi A. Microimmiscibility and three-dimensional dynamic structures of phosphatidylcholine-cholesterol membranes: translational diffusion of a copper complex in the membrane. *Biochemistry*. 1990;29(34):7936-7945. doi:10.1021/bi00486a023.

95. Subczynski WK, Hyde JS, Kusumi A. Effect of alkyl chain unsaturation and cholesterol intercalation on oxygen transport in membranes: a pulse ESR spin labeling study. *Biochemistry*. 1991;30(35):8578-8590. doi:10.1021/bi00099a013.

96. Pasenkiewicz-Gierula M, Subczynski WK, Kusumi A. Influence of phospholipid unsaturation on the cholesterol distribution in membranes. *Biochimie*. 1991;73(10):1311-1316. doi:10.1016/0300-9084(91)90094-h.

97. Mouritsen OG. The liquid-ordered state comes of age. *Biochim Biophys Acta*. 2010;1798(7):1286-1288. doi:10.1016/j.bbamem.2010.02.017.

98. Veatch SL, Keller SL. Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys J*. 2003;85(5):3074-3083. doi:10.1016/S0006-3495(03)74726-2.

99. Yetukuri L, Ekroos K, Vidal-Puig A, Oresic M. Informatics and computational strategies for the study of lipids. *Mol Biosyst*. 2008;4(2):121-127. doi:10.1039/b715468b.

100. Kusumi A, Sako Y. Cell surface organization by the membrane skeleton. *Curr Opin Cell Biol*. 1996;8(4):566-574. doi:10.1016/s0955-0674(96)80036-6.

101. Kusumi A, Nakada C, Ritchie K, et al. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct*. 2005;34(1):351-378. doi:10.1146/annurev.biophys.34.040204.144637.

102. Morone N, Fujiwara T, Murase K, et al. Three-dimensional reconstruction of the membrane skeleton at the plasma membrane interface by electron tomography. *J Cell Biol*. 2006;174(6):851-862. doi:10.1083/jcb.200606007.

103. Han B, Zhou R, Xia C, Zhuang X. Structural organization of the actin-spectrin-based membrane skeleton in dendrites and soma of neurons. *Proc Natl Acad Sci USA*. 2017;114(32):E6678-E6685. doi:10.1073/pnas.1705043114.

104. Sengupta P, Hammond A, Holowka D, Baird B. Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles. *Biochim Biophys Acta*. 2008;1778(1):20-32. doi:10.1016/j.bbamem.2007.08.028.

105. Bauer B, Davidson M, Orwar O. Proteomic analysis of plasma membrane vesicles. *Angew Chem Int Ed Engl*. 2009;48(9):1656-1659. doi:10.1002/anie.200803898.

106. Holowka D, Baird B. Structural studies on the membrane-bound immunoglobulin E-receptor complex. 1. Characterization of large plasma membrane vesicles from rat basophilic leukemia cells and insertion of amphipathic fluorescent probes. *Biochemistry*. 1983;22(14):3466-3474. doi:10.1021/bi00283a025.

107. Gidwani A, Holowka D, Baird B. Fluorescence anisotropy measurements of lipid order in plasma membranes and lipid rafts from RBL-2H3 mast cells. *Biochemistry*. 2001;40(41):12422-12429. doi:10.1021/bi010496c.

108. Sezgin E, Kaiser H-J, Baumgart T, Schwille P, Simons K, Levental I. Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat Protoc*. 2012;7(6):1042-1051. doi:10.1038/nprot.2012.059.

109. Johnson SA, Stinson BM, Go MS, et al. Temperature-dependent phase behavior and protein partitioning in giant plasma membrane vesicles. *Biochim Biophys Acta*. 2010;1798(7):1427-1435. doi:10.1016/j.bbamem.2010.03.009.

110. Levental I, Lingwood D, Grzybek M, Coskun Ü, Simons K. Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proc Natl Acad Sci USA*. 2010;107(51):22050-22054. doi:10.1073/pnas.1016184107.

111. Kusumi A, Tsuda M, Akino T, Ohnishi S, Terayama Y. Protein-phospholipid-cholesterol interaction in the photolysis of invertebrate rhodopsin. *Biochemistry*. 1983;22(5):1165-1170. doi:10.1021/bi00274a027.

112. LaRocca TJ, Pathak P, Chiantia S, et al. Proving lipid rafts exist: membrane domains in the prokaryote Borrelia burgdorferi have the same properties as eukaryotic lipid rafts. Coburn J, ed. *PLoS Pathog*. 2013;9(5):e1003353. doi:10.1371/journal.ppat.1003353.

113. Schuberth C, Wedlich-Soldner R. Building a patchwork - The yeast plasma membrane as model to study lateral domain formation. *Biochim Biophys Acta*. 2015;1853(4):767-774. doi:10.1016/j.bbamcr.2014.12.019.

114. Rayermann SP, Rayermann GE, Cornell CE, Merz AJ, Keller SL. Hallmarks of Reversible Separation of Living, Unperturbed Cell Membranes into Two Liquid Phases. *Biophys J*. 2017;113(11):2425-2432. doi:10.1016/j.bpj.2017.09.029.

115. Ge M, Gidwani A, Brown HA, Holowka D, Baird B, Freed JH. Ordered and disordered phases coexist in plasma membrane vesicles of RBL-2H3 mast cells. An ESR study. *Biophys J*. 2003;85(2):1278-1288. doi:10.1016/S0006-3495(03)74563-9.

116. Viola A, Gupta N. Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. *Nat Rev Immunol*. 2007;7(11):889-896. doi:10.1038/nri2193.

117. Rao M, Mayor S. Active organization of membrane constituents in living cells. *Curr Opin Cell Biol*. 2014;29:126-132. doi:10.1016/j.ceb.2014.05.007.

118. Köster DV, Mayor S. Cortical actin and the plasma membrane: inextricably intertwined. *Curr Opin Cell Biol*. 2016;38:81-89. doi:10.1016/j.ceb.2016.02.021.

119. Honerkamp-Smith AR, Cicuta P, Collins MD, et al. Line tensions, correlation lengths,

and critical exponents in lipid membranes near critical points. *Biophys J*. 2008;95(1):236-246. doi:10.1529/biophysj.107.128421.

120. Veatch SL, Pietro Cicuta, Sengupta P, Honerkamp-Smith A, Holowka D, Baird B. Critical fluctuations in plasma membrane vesicles. *ACS Chem Biol*. 2008;3(5):287-293. doi:10.1021/cb800012x.

121. Machta BB, Veatch SL, Sethna JP. Critical Casimir forces in cellular membranes. *Phys Rev Lett*. 2012;109(13):138101. doi:10.1103/PhysRevLett.109.138101.

122. Kusumi A, Hyde JS. Spin-label saturation-transfer electron spin resonance detection of transient association of rhodopsin in reconstituted membranes. *Biochemistry*. 1982;21(23):5978-5983. doi:10.1021/bi00266a039.

123. Raghunathan K, Wong TH, Chinnapen DJ, Lencer WI, Jobling MG, Kenworthy AK. Glycolipid Crosslinking Is Required for Cholera Toxin to Partition Into and Stabilize Ordered Domains. *Biophys J*. 2016;111(12):2547-2550. doi:10.1016/j.bpj.2016.11.008.

124. Mondal M, Mesmin B, Mukherjee S, Maxfield FR. Sterols are mainly in the cytoplasmic leaflet of the plasma membrane and the endocytic recycling compartment in CHO cells. *Mol Biol Cell*. 2009;20(2):581-588. doi:10.1091/mbc.e08-07-0785.

125. Wüstner D, Modzel M, Lund FW, Lomholt MA. Imaging approaches for analysis of cholesterol distribution and dynamics in the plasma membrane. *Chem Phys Lipids*. 2016;199:106-135. doi:10.1016/j.chemphyslip.2016.03.003.

126. Ariola FS, Li Z, Cornejo C, Bittman R, Heikal AA. Membrane fluidity and lipid order in ternary giant unilamellar vesicles using a new bodipy-cholesterol derivative. *Biophys J*. 2009;96(7):2696-2708. doi:10.1016/j.bpj.2008.12.3922.

127. Kiessling V, Crane JM, Tamm LK. Transbilayer Effects of Raft-Like Lipid Domains in Asymmetric Planar Bilayers Measured by Single Molecule Tracking. *Biophys J*. 2005;91(9):3313-3326. doi:10.1529/biophysj.106.091421.

128. Blosser MC, Honerkamp-Smith AR, Han T, Haataja M, Keller SL. Transbilayer Colocalization of Lipid Domains Explained via Measurement of Strong Coupling Parameters. *Biophys J*. 2015;109(11):2317-2327. doi:10.1016/j.bpj.2015.10.031.

129. Raghupathy R, Anilkumar AA, Polley A, et al. Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell*. 2015;161(3):581-594. doi:10.1016/j.cell.2015.03.048.

130. Lipowsky R. Remodeling of membrane compartments: some consequences of membrane fluidity. *Biol Chem*. 2014;395(3):253-274. doi:10.1515/hsz-2013-0244.

131. Bassereau P, Jin R, Baumgart T, et al. The 2018 biomembrane curvature and remodeling roadmap. *J Phys D: Appl Phys*. 2018;51(34):343001. doi:10.1088/1361-6463/aacb98.

132. Liu Y, Lipowsky R, Dimova R. Giant Vesicles Encapsulating Aqueous Two-Phase Systems: From Phase Diagrams to Membrane Shape Transformations. *Front Chem*. 2019;7:213. doi:10.3389/fchem.2019.00213.

133. Yang S-T, Kreutzberger AJB, Kiessling V, Ganser-Pornillos BK, White JM, Tamm LK. HIV virions sense plasma membrane heterogeneity for cell entry. *Sci Adv*. 2017;3(6):e1700338–. doi:10.1126/sciadv.1700338.

134. Seno K, Hayashi F. Palmitoylation is a prerequisite for dimerization-dependent raftophilicity of rhodopsin. *J Biol Chem*. 2017;292(37):15321-15328. doi:10.1074/jbc.M117.804880.

135. Hayashi F, Saito N, Tanimoto Y, et al. Raftophilic rhodopsin-clusters offer stochastic platforms for G protein signalling in retinal discs. *Commun Biol*. 2019;2(1):209. doi:10.1038/s42003-019-0459-6.

136. Coskun Ü, Grzybek M, Drechsel D, Simons K. Regulation of human EGF receptor by lipids. *Proc Natl Acad Sci USA*. 2011;108(22):9044-9048. doi:10.1073/pnas.1105666108.

137. Montixi C, Langlet C, Bernard AM, et al. Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J*. 1998;17(18):5334-5348. doi:10.1093/emboj/17.18.5334.

138. Xavier R, Brennan T, Li Q, McCormack C, Seed B. Membrane Compartmentation Is Required for Efficient T Cell Activation. *Immunity*. 1998;8(6):723-732. doi:10.1016/S1074-7613(00)80577-4.

139. Davey A, Liu W, Sohn HW, Brzostowski J, Pierce SK. Understanding the initiation of B cell signaling through live cell imaging. *Meth Enzymol*. 2012;506:265-290. doi:10.1016/B978-0-12-391856-7.00038-X.

140. Molnár E, Swamy M, Holzer M, et al. Cholesterol and sphingomyelin drive ligand-independent T-cell antigen receptor nanoclustering. *J Biol Chem*. 2012;287(51):42664-42674. doi:10.1074/jbc.M112.386045.

141. Beck-García K, Beck-García E, Bohler S, et al. Nanoclusters of the resting T cell antigen receptor (TCR) localize to non-raft domains. *Biochim Biophys Acta*. 2015;1853(4):802-809. doi:10.1016/j.bbamcr.2014.12.017.

142. van Zanten TS, Gómez J, Manzo C, et al. Direct mapping of nanoscale compositional connectivity on intact cell membranes. *Proc Natl Acad Sci USA*. 2010;107(35):15437-

15442. doi:10.1073/pnas.1003876107.

143. Bakker GJ, Eich C, Torreno-Piña JA, et al. Lateral mobility of individual integrin nanoclusters orchestrates the onset for leukocyte adhesion. *Proc Natl Acad Sci USA*. 2012;109(13):4869-4874. doi:10.1073/pnas.1116425109.

144. Yamaji A, Sekizawa Y, Emoto K, et al. Lysenin, a novel sphingomyelin-specific binding protein. *J Biol Chem*. 1998;273(9):5300-5306. doi:10.1074/jbc.273.9.5300.

145. Itoh T, Takenawa T. Phosphoinositide-binding domains: Functional units for temporal and spatial regulation of intracellular signalling. *Cell Signal*. 2002;14(9):733-743. doi:10.1016/s0898-6568(02)00028-1.

146. Furutani M, Tsujita K, Itoh T, Ijuin T, Takenawa T. Application of phosphoinositide-binding domains for the detection and quantification of specific phosphoinositides. *Analytical Biochemistry*. 2005;355(1):8-18. doi:10.1016/j.ab.2006.05.014.

147. Ohno-Iwashita Y, Shimada Y, Hayashi M, Iwamoto M, Iwashita S, Inomata M. Cholesterol-binding toxins and anti-cholesterol antibodies as structural probes for cholesterol localization. *Subcell Biochem*. 2010;51:597-621. doi:10.1007/978-90-481-8622-8_22.

148. Abe M, Kobayashi T. Imaging local sphingomyelin-rich domains in the plasma membrane using specific probes and advanced microscopy. *Biochim Biophys Acta*. 2014;1841(5):720-726. doi:10.1016/j.bbalip.2013.07.003.

149. Maekawa M, Fairn GD. Molecular probes to visualize the location, organization and dynamics of lipids. *J Cell Sci*. 2014;127(Pt 22):4801-4812. doi:10.1242/jcs.150524.

150. Nelson LD, Johnson AE, London E. How interaction of perfringolysin O with membranes is controlled by sterol structure, lipid structure, and physiological low pH: insights into the origin of perfringolysin O-lipid raft interaction. *J Biol Chem*. 2008;283(8):4632-4642. doi:10.1074/jbc.M709483200.

151. Das A, Goldstein JL, Anderson DD, Brown MS, Radhakrishnan A. Use of mutant 125I-perfringolysin O to probe transport and organization of cholesterol in membranes of animal cells. *Proc Natl Acad Sci USA*. 2013;110(26):10580-10585. doi:10.1073/pnas.1309273110.

152. Makino A, Abe M, Ishitsuka R, et al. A novel sphingomyelin/cholesterol domain-specific probe reveals the dynamics of the membrane domains during virus release and in Niemann-Pick type C. *FASEB J*. 2017;31(4):1301-1322. doi:10.1096/fj.201500075R.

153. Endapally S, Frias D, Grzemska M, Gay A, Tomchick DR, Radhakrishnan A. Molecular Discrimination between Two Conformations of Sphingomyelin in Plasma Membranes.

Cell. 2019;176(5):1040–1053.e17. doi:10.1016/j.cell.2018.12.042.

154. Kenworthy AK, Nichols BJ, Remmert CL, et al. Dynamics of putative raft-associated proteins at the cell surface. *J Cell Biol*. 2004;165(5):735-746. doi:10.1083/jcb.200312170.

155. Chapple JP, Grayson C, Hardcastle AJ, et al. Organization on the plasma membrane of the retinitis pigmentosa protein RP2: investigation of association with detergent-resistant membranes and polarized sorting. *Biochem J*. 2003;372(Pt 2):427-433. doi:10.1042/BJ20021475.

156. Drevot P, Langlet C, Guo X-J, et al. TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts. *EMBO J*. 2002;21(8):1899-1908. doi:10.1093/emboj/21.8.1899.

157. Dietrich C, Bagatolli LA, Volovyk ZN, et al. Lipid Rafts Reconstituted in Model Membranes. *Biophys J*. 2001;80(3):1417-1428. doi:10.1016/S0006-3495(01)76114-0.

158. Dietrich C, Volovyk ZN, Levi M, Thompson NL, Jacobson K. Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc Natl Acad Sci USA*. 2001;98(19):10642-10647. doi:10.1073/pnas.191168698.

159. Seeliger J, Erwin N, Rosin C, Kahse M, Weise K, Winter R. Exploring the structure and phase behavior of plasma membrane vesicles under extreme environmental conditions. *Phys Chem Chem Phys*. 2015;17(11):7507-7513. doi:10.1039/c4cp05845c.

160. Honigmann A, Mueller V, Hell SW, Eggeling C. STED microscopy detects and quantifies liquid phase separation in lipid membranes using a new far-red emitting fluorescent phosphoglycerolipid analogue. *Faraday Discuss*. 2013;161:77-89. doi:10.1039/C2FD20107K.

161. Wang TY, Silvius JR. Different sphingolipids show differential partitioning into sphingolipid/cholesterol-rich domains in lipid bilayers. *Biophys J*. 2000;79(3):1478-1489. doi:10.1016/S0006-3495(00)76399-5.

162. Ramadurai S, Holt A, Krasnikov V, van den Bogaart G, Killian JA, Poolman B. Lateral diffusion of membrane proteins. *J Am Chem Soc*. 2009;131(35):12650-12656. doi:10.1021/ja902853g.

163. Saffman PG, Delbrück M. Brownian motion in biological membranes. *Proc Natl Acad Sci USA*. 1975;72(8):3111-3113. doi:10.1073/pnas.72.8.3111.

164. Iino R, Koyama I, Kusumi A. Single Molecule Imaging of Green Fluorescent Proteins in Living Cells: E-Cadherin Forms Oligomers on the Free Cell Surface. *Biophys J*. 2001;80(6):2667-2677. doi:10.1016/S0006-3495(01)76236-4.

165. Gambin Y, Lopez-Esparza R, Reffay M, et al. Lateral mobility of proteins in liquid membranes revisited. *Proc Natl Acad Sci USA*. 2006;103(7):2098-2102. doi:10.1073/pnas.0511026103.

166. Zhou Y, Maxwell KN, Sezgin E, et al. Bile acids modulate signaling by functional perturbation of plasma membrane domains. *J Biol Chem*. 2013;288(50):35660-35670. doi:10.1074/jbc.M113.519116.

167. Agudo-Ibáñez L, Herrero A, Barbacid M, Crespo P. H-ras distribution and signaling in plasma membrane microdomains are regulated by acylation and deacylation events. *Mol Cell Biol*. 2015;35(11):1898-1914. doi:10.1128/MCB.01398-14.

168. Kahya N, Schwille P. Fluorescence correlation studies of lipid domains in model membranes. *Mol Membr Biol*. 2006;23(1):29-39. doi:10.1080/09687860500489099.

169. Field KA, Holowka D, Baird B. Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc Natl Acad Sci USA*. 1995;92(20):9201-9205.

170. Field KA, Holowka D, Baird B. Compartmentalized Activation of the High Affinity Immunoglobulin E Receptor within Membrane Domains. *J Biol Chem*. 1997;272(7):4276-4280. doi:10.1074/jbc.272.7.4276.

171. Field KA, Holowka D, Baird B. Structural aspects of the association of FcepsilonRI with detergent-resistant membranes. *J Biol Chem*. 1999;274(3):1753-1758. doi:10.1074/jbc.274.3.1753.

172. Webb Y, Hermida-Matsumoto L, Resh MD. Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J Biol Chem*. 2000;275(1):261-270. doi:10.1074/jbc.275.1.261.

173. Prior IA, Harding A, Yan J, Sluimer J, Parton RG, Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. *Nat Cell Biol*. 2001;3(4):368-375. doi:10.1038/35070050.

174. Chen X, Jen A, Warley A, Lawrence MJ, Quinn PJ, Morris RJ. Isolation at physiological temperature of detergent-resistant membranes with properties expected of lipid rafts: the influence of buffer composition. *Biochem J*. 2009;417(2):525-533. doi:10.1042/BJ20081385.

175. Morris RJ. Ionic control of the metastable inner leaflet of the plasma membrane: Fusions natural and artefactual. *FEBS Lett*. 2010;584(9):1665-1669. doi:10.1016/j.febslet.2009.11.017.

176. Saslowsky DE, Lawrence J, Ren X, Brown DA, Henderson RM, Edwardson JM. Placental alkaline phosphatase is efficiently targeted to rafts in supported lipid

bilayers. *J Biol Chem*. 2002;277(30):26966-26970. doi:10.1074/jbc.M204669200.

177. Lorent JH, Levental I. Structural determinants of protein partitioning into ordered membrane domains and lipid rafts. *Chem Phys Lipids*. 2015;192:23-32. doi:10.1016/j.chemphyslip.2015.07.022.

178. Hammond AT, Heberle FA, Baumgart T, Holowka D, Baird B, Feigenson GW. Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc Natl Acad Sci USA*. 2005;102(18):6320-6325. doi:10.1073/pnas.0405654102.

179. Chinnapen DJF, Hsieh W-T, Welscher te YM, et al. Lipid sorting by ceramide structure from plasma membrane to ER for the cholera toxin receptor ganglioside GM1. *Dev Cell*. 2012;23(3):573-586. doi:10.1016/j.devcel.2012.08.002.

180. Spiegel S, Kassis S, Wilchek M, Fishman PH. Direct visualization of redistribution and capping of fluorescent gangliosides on lymphocytes. *J Cell Biol*. 1984;99(5):1575-1581. doi:10.1083/jcb.99.5.1575.

181. Spiegel S. Fluorescent derivatives of ganglioside GM1 function as receptors for cholera toxin. *Biochemistry*. 1985;24(21):5947-5952. doi:10.1021/bi00342a039.

182. Goins B, Masserini M, Barisas BG, Freire E. Lateral diffusion of ganglioside GM1 in phospholipid bilayer membranes. *Biophys J*. 1986;49(4):849-856. doi:10.1016/S0006-3495(86)83714-6.

183. Marushchak D, Gretskaya N, Mikhalyov I, Johansson LBÅ. Self-aggregation--an intrinsic property of G(M1) in lipid bilayers. *Mol Membr Biol*. 2007;24(2):102-112. doi:10.1080/09687860600995235.

184. Mikhalyov I, Gretskaya N, Johansson LBÅ. Fluorescent BODIPY-labelled GM1 gangliosides designed for exploring lipid membrane properties and specific membrane-target interactions. *Chem Phys Lipids*. 2009;159(1):38-44. doi:10.1016/j.chemphyslip.2009.01.005.

185. Šachl R, Amaro M, Aydogan G, et al. On multivalent receptor activity of GM1 in cholesterol containing membranes. *Biochim Biophys Acta*. 2015;1853(4):850-857. doi:10.1016/j.bbamcr.2014.07.016.

186. Scheidt HA, Muller P, Herrmann A, Huster D. The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol. *J Biol Chem*. 2003;278(46):45563-45569. doi:10.1074/jbc.M303567200.

187. Zhang Y, Bulkley DP, Xin Y, et al. Structural Basis for Cholesterol Transport-like Activity of the Hedgehog Receptor Patched. *Cell*. 2018;175(5):1352–1364.e14. doi:10.1016/j.cell.2018.10.026.

Figure Legends

Figure 1. GUVs consisting of the ternary mixture of SM(18:0)/DOPC/cholesterol (1:1:1 by moles) labeled with proper fluorescent lipid pairs exhibited micron-scale two complementary domains at 28.5˚C, suggesting the occurrence of phase separation. (Top) 594neg-SM(18:0) and DPPC-C5-BodipyFL (Acyl). (Bottom) 488neg-SM(18:0) and 594neg-DOPC. The structures of the phospholipid analogs with a neg linker are shown in Fig. 2. In the images of 594neg-SM(18:0) and 488neg-SM(18:0), note that both of the two domains are labeled, with different intensities, by a factor of about 4. This result is consistent with the observations made with deuterated SM(18:0), suggesting that these fluorescent SM analogs behave very much like their parental molecules. Bars, 20 μm. Adapted from Fig. 2B of Ref. 31 with permission. The probes' chemical names are provided in the notes to Table 1.

Figure 2. Chemical structures of fluorescent sphingomyelin (SM) and PC analogs that have been shown to behave in ways very similar to their non-labeled endogenous molecules. These analogs keep the same charges on the phosphate and choline groups as their parental molecules, due to the addition of the fluorescent probes to the choline group of SM and PC by using Huisgen cycloaddition (red), and the fluorescent probes are placed quite far away from the choline headgroup of the parental lipid by using a hydrophilic nonaethylene glycol (neg) linker. For comparison, the chemical structure of a DOPE probe often used as an Ld marker is shown. The fluorescent probe is directly bound to the amine group, and thus the positive charge there is lost and the fluorescent probe is placed close to the membrane surface. 488 and 594 are ATTO488 and ATTO594 dyes, respectively. Since these fluorescent phospholipid analogs and the fluorescent ganglioside analogs that behave very much like their parental gangliosides (Fig. 4) have been developed quite recently by our group and are not found in literature very easily, their chemical structures are shown. Adapted from Fig. 1 of Ref. 31 with permission.

Figure 3. GPMVs, prepared from RBL-2H3 cells, labeled with proper fluorescent lipid pairs, and then cooled to 10˚C, exhibit two coexisting and complementarily-labeled micron-scale domains

(greater than the optical diffraction limit of ~200 nm). (Top) DPPC-C5-BodipyFL (Acyl) and 594neg-SM(18:0). (Bottom) 488neg-SM(18:0) and 594neg-DOPC. Adapted from Fig. 5A of Ref. 31 with permission.

Figure 4. Chemical structures of the fluorescent analogs of the gangliosides GM1, GM2, GM3, and GD1b entirely produced by chemical synthesis. Reproduced from Fig. 1 of Ref. 30 with permission.