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Phase Separation in Lipid Membranes

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Cell membranes show complex behavior, in part because of the large number of different components that interact with each other in different ways. One aspect of this complex behavior is lateral organization of components on a range of spatial scales. We found that lipid-only mixtures can model the range of size scales, from approximately 2 nm up to microns. Furthermore, the size of compositional heterogeneities can be controlled entirely by lipid composition for mixtures such as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/cholesterol or sphingomyelin (SM)/DOPC/POPC/cholesterol. In one region of special interest, because of its connection to cell membrane rafts, nanometer-scale domains of liquid-disordered phase and liquid-ordered phase coexist over a wide range of compositions.

SCALES OF SPATIAL ORGANIZATION OF CELL MEMBRANES

Cell membranes have daunting complexity, including but not limited to the scales of spatial organization that emerge from a complex system. Complex behavior arises in part from the large number of different kinds of lipids and proteins, the different ways in which these membrane components interact with each other and with the rest of the cell, and the dynamic processes that locally and dramatically change significant fractions of the membrane. To appreciate the nature of this behavior, various key aspects of the complexity must be examined. Although a complete picture has not yet emerged, the available data are compelling that compositional heterogeneity, rather than random mixing of membrane lipids and proteins, describes cell membranes (Lingwood and Simons 2010). Here, we focus on one specific subset of complex membrane behavior: lateral heterogeneity based on lipid–lipid interactions in multicomponent bilayer mixtures. The impetus for examining such mixtures is the apparent connection between the coexistence of liquid-disordered (Ld) and liquid-ordered (Lo) phases observed in some simple, lipid-only bilayers, and the properties of lipid rafts observed in some cellular membranes. One possible starting point for experimental study is to find the simplest system that shows a wide range of organization comparable to what is found in cells, that is, from nanometers to microns. This turns out

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to be a four-component lipid bilayer mixture at equilibrium, containing cholesterol.

Membranes of living cells show spatial organization on all relevant size scales, ranging from molecular lengths to the dimensions of the cell itself. The plasma membrane is the site of controlled crossing of molecules and ions between the cell and the environment, and is the anisotropic medium in which proteins find their partners and catalyze chemical reactions. The size scale for such molecular interactions is in the range of a few nanometers. Some larger bilayer environments are known to have specialized purposes, for example, membrane regions on the order of 50–100 nm that characterize caveolae and other sites of membrane fusion and endocytosis. A larger, micron-size scale describes the clustering of proteins and lipids in the membrane site of an immune synapse (Manz and Groves 2010). At a scale of tens of microns, epithelial cell plasma membranes are organized into the very different morphological and compositional regions of apical and basolateral domains (Simons and van Meer 1988).

Small size scales of organization in a cell membrane are difficult to characterize experimentally. Several techniques have proven useful for detecting heterogeneities on the scale of such small collections of molecules, although not providing quantitative size measurement. Methods that are sensitive to the immediate environment of a molecule are probing a very short spatial scale, ~1 nm. Fluorescence techniques including anisotropy (Kinosita et al. 1984), lifetime (Haluska et al. 2008), quantum yield (Zhao et al. 2007a), and quenching by spin-labels (London and Feigenson 1981), as well as the ESR spectrum itself (Schneider and Freed 1989), are such highly locally sensitive methods. Although first-order phase separation creates different environments on both small and large spatial scales, simple nonideal lipid mixing also creates different local environments at the ~1 nm scale. Experimental sensitivity to such small heterogeneities is needed, along with quantitative measure of spatial scale and (if possible) lifetime of any such domains. Super-resolution imaging is highly promising in this regard. In a recent study, STED microscopy of fluorescently labeled lipids revealed transient trapping of sphingomyelin for an average time of ~15 msec within regions of diameter <20 nm (Sahl et al. 2010). Of course, these STED-detected domains themselves must last longer than 15 msec. This highly significant finding rules out simple nonideal mixing, which would result in molecular clusters that dissipate on a timescale of tens of microseconds (Abney and Scalettar 1995). In addition to techniques employing a single probe species, FRET between donor and acceptor fluorophores is in principle capable of providing a measure of domain size in the range $1 - 10 \times R_0$, which is in the range of 2–80 nm for available membrane fluorescent probes (Feigenson and Buboltz 2001). Finally, we note that any technique involving addition of an extrinsic probe carries the caveat that the probe molecule might perturb the native spatial organization of the membrane.

MODELING SPATIAL ORGANIZATION IN CELL MEMBRANES

The size scales, time scales, and patterns of heterogeneity found in membranes of live cells can be treated in terms of general physical principles (Seul and Andelman 1995; Imperio and Reatto 2006; Elson et al. 2010; Fan and Sammalkorpi 2010). One way to organize such general considerations is to classify the driving forces as equilibrium or nonequilibrium processes. For separated phases at equilibrium, competing interactions can give rise to patterns of spatial organization with variations in size, lifetime, and morphology. For example, when line tension dominates any other interactions that depend on domain (perimeter) size, coexisting liquid phases round up into large circular domains containing vast numbers of molecules to minimize their perimeter. This simple morphology can be modulated when interactions of sufficient magnitude compete with line tension, so that patterns appear—a maze of stripes, tiny round domains, curved lines, and branched lines (Seul and Andelman 1995). Also at equilibrium, and depending on proximity to a critical point, fluctuating domains showing a range of size and timescales appear (Honerkamp-Smith et al. 2008).

Processes occurring far from equilibrium can strongly influence the nature of coexisting domains. An especially simple case is the failure of phase-separated Ld + Lo macroscopic domains to merge into an equilibrium large domain because of a high-energy barrier from membrane curvature at the domain boundaries (Semrau et al. 2009). Nanodomains can also be stabilized by entropy: If line tension is sufficiently low, the entropic penalty for merging many small domains into a single large domain can produce a "quasi-equilibrium" state of domains smaller than 50 nm (Frolov et al. 2006). A more complex and interesting process, also far from equilibrium and connected to other events in the cell, is the removal and delivery of patches of membrane having a composition distinct from that of the larger membrane. This dynamic process can generate a wide range of domain sizes and morphologies (Fan and Sammalkorpi 2010).

LIPIDS FOUND IN CELL MEMBRANES

Considering only the lipids (a mixture chosen by evolution in part to spontaneously form the bilayer phases that are the fundamental cell membrane structure), a first step for study of heterogeneity is to find the lipid composition of cell membranes (van Meer et al. 2008). A complete analysis would involve assaying the lipids of the given cell membrane, both qualitatively and quantitatively, and finding the particular composition of each leaflet. The latter has been a vexing problem for decades. Whole membrane assays are more feasible, and large-scale efforts from "lipidomics" initiatives (Wenk 2005) have been successful in purifying particular cell membranes and measuring their overall composition, albeit without being able to characterize separate leaflet compositions so far. Different types of lipids have been found to predominate in different organisms, and in different membranes within a given cell. Even with so much new information, the connection between events of cell biology and particular lipid compositions—a kind of "lipid role assignment"—can be made in but a few cases. These exceptions include the phosphoinositide binding of particular proteins to the plasma membrane inner leaflet (McLaughlin and Murray 2005) and polyunsaturated acyl chains of lipids in the retinal rod, which are needed for full activity of rhodopsin (Polozova and Litman 2000). More information is known about plasma membranes than about any other cell membranes, making the plasma membrane a starting point for thinking about lipid roles in mammalian cell membranes.

In the favorable case that a particular membrane can be obtained in high purity, the overall lipid composition can be found. In comparison with the total lipids of a cell, two categories of lipids stand out for their abundance in the plasma membrane compared with other cell membranes: high melting temperature lipids, in particular sphingomyelin and gangliosides, and cholesterol (van Meer et al. 2008). These types of lipids are always present in the plasma membrane along with much lower melting temperature lipids, which in the outer leaflet are mostly phosphatidylcholines (Gennis 1989). After so many years of effort, it is perhaps surprising that the precise lipid composition has not been separately determined for outer and inner leaflets for any plasma membrane. Because the best understood case is that of the mature human erythrocyte, we will take these results as an approximate model for other mammalian plasma membranes: the outer leaflet has all of the gangliosides, the great majority of the sphingomyelin (SM), and much of the phosphatidylcholine (PC) and cholesterol; the inner leaflet has most of the membrane's phosphatidylethanolamine (PE), possibly all of its phosphatidylserine (PS) and phosphatidylinositol (PI) and some PC, and much cholesterol (Gennis 1989). It is important to remember that these numbers reflect an average over the entire membrane. Even if we know the composition of a leaflet, this does not imply that exactly this composition is found at all locations over the entire membrane surface, (e.g., apical and basolateral regions of an epithelial cell). In addition, many other lipid components, including the plasmalogen versions of glycerophospholipids, are also present. The phase behavior of these molecules in mixtures with other membrane components is largely unexplored.

60,000 lipids per leaflet. A domain of this size is certainly large enough to be considered a phase, or even to have coexisting phases within the domain. But what is the nature of a 5 nm patch containing only approximately 40 molecules?

The thermodynamics of mixing, together with Monte Carlo computer simulations, provides a powerful, systematic basis for examination of lipid distributions based on lipid–lipid interaction energies. A simple visual inspection of Monte Carlo snapshots at equilibrium, such as those shown in Figures 1 and 2, is instructive. Cell membranes are far from random mixtures of lipids and proteins, but we start by considering exactly this case: an ideal mixture of, for example, phosphatidylcholines, defined by an excess mixing energy $\Delta Em = 0$ (Guggenheim 1952). Figure 1A shows that, for an ideal equimolar binary mixture with only nearest-neighbor interactions (e.g., no long-range or higher order interactions), groupings of three to five like lipids in each leaflet are commonly observed (Huang and Feigenson 1993). First-order phase separation occurs when ΔEm exceeds 0.55 kT (Guggenheim 1952), and results in large phase domains seen in Figure 1D,E. Intermediate values of ΔEm generate small clusters. For example, with $\Delta Em = 0.4$ kT, the sizes of the numerous clusters are in the range 10 to 20 lipids (see Fig. 1B), a "domain size" of ~3 nm (Huang and Feigenson 1993). At $\Delta Em = 0.5$ kT, numerous clusters of approximately 100 lipids form, as shown in Figure 1C,F. Such small clusters from nonideal mixing have a short lifetime on the order of microseconds, disappearing and forming again elsewhere after many nearest-neighbor exchanges, each $\sim 10^{-7}$ s (Abney and Scalettar 1995).

Further insight into lipid mixing comes from considering the more complex behavior of multicomponent bilayer mixtures. Figure 2 shows snapshots of mixing behavior in which the interaction energies are held constant, but the concentration of a third component is

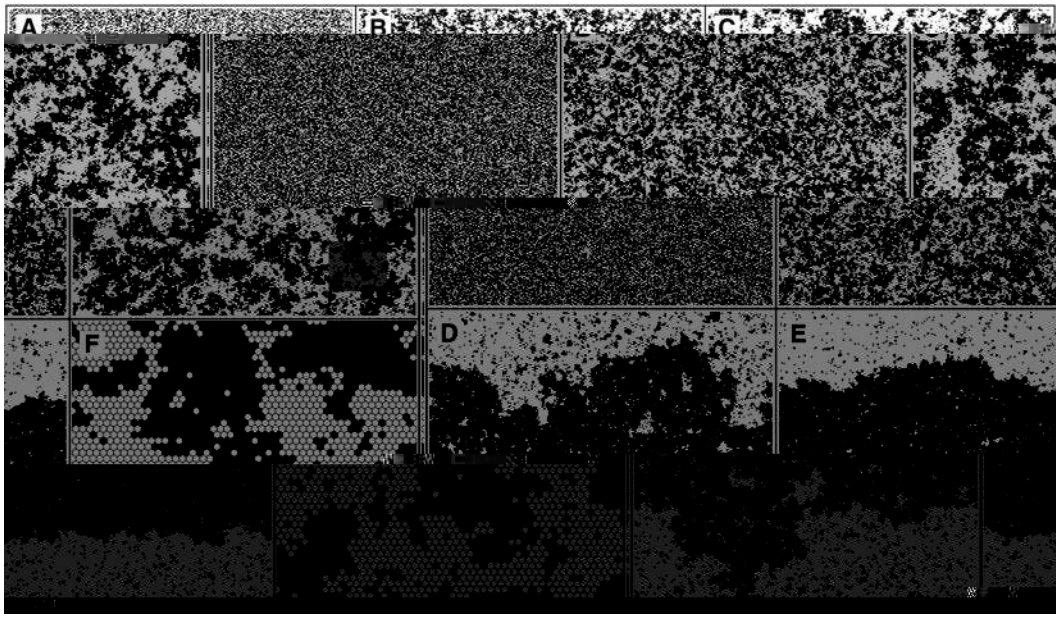

Figure 1. Lattice snapshots for Monte Carlo simulations of binary mixtures with different pairwise interaction energy ΔEm. (A) A random mixture ($\Delta Em = 0$) is characterized by groupings of 3 to 5 lipids. (B) An unfavorable interaction of $\Delta Em = 0.4$ kT results in clusters of 10 to 20 lipids, or a domain size of ~3 nm. (C) At $\Delta Em = 0.5$ kT the system is close to phase separation. An enlargement of the snapshot (F) reveals large clusters composed of hundreds of lipids. (D) At $\Delta Em = 0.55$ kT the clusters coalesce, indicating a phase transition. (E) $\Delta Em = 0.6$ kT. The phase-separated mixture is characterized by a single domain of each phase.

Figure 2. Lattice snapshots for Monte Carlo simulations of ternary mixtures. (E) An equimolar binary α/β mixture with an unfavorable pairwise interaction energy $\Delta E_{\alpha\beta} = 0.8kT$ is characterized by coexistence of α-rich and β-rich phases. (A–D) Although maintaining the ratio of components α and β, a third component, γ (which could be cholesterol), is added that interacts favorably with both α and β ($\Delta E_{\alpha\gamma} = -0.8$ kT, $\Delta E_{\beta\gamma} = -1.2$ kT). (D) At $\chi_\gamma = 0.20$, large clusters of β within the α-rich phase (and vice versa) are evident. (C) Long-range structure is broken up at $\chi_\gamma = 0.25$. An enlargement of the snapshot (F) shows clusters of hundreds of lipids, and the uneven distribution of component γ (gray) between α-rich (white) and β-rich (black) clusters. Further addition of component γ reduces the size of clusters. (B) Snapshot for $\chi_\gamma = 0.30$ and (A) $\chi_\gamma = 0.35$.

varied. For comparison with Figure 1, Monte Carlo simulation snapshots shown in Figure 2 of a three-component mixture are arranged in an order that leads up to phase separation in Figure 2E. In these simulations, increasing cholesterol concentration leads to increased mixing of the components. At a cholesterol concentration of 35 mol % the mixture shows highly nonrandom mixing, with clusters of approximately 20 lipids (Fig. 2A). Smaller cholesterol concentrations lead to increasing cluster sizes. For example, Figure 2C,F show clusters of several hundreds of lipids at 25 mol % cholesterol, corresponding to a domain size of approximately 10 nm.

Considering these two simulation studies of two- and three-component mixtures, what do we learn about the spatial distribution of lipids? First, even well-mixed or random mixtures show numerous clusters of 3 to 5 similar lipids. Second, lipid clusters grow as the mixture is changed to approach a boundary of phase separation, but only in a limited way: Cluster sizes approach a maximal range that is similar for the mixtures with different numbers of components. However, a short distance past the phase boundary in composition space (Fig. 2) or in energy (Fig. 1) results in enormous, abrupt change in size of domains.

Structured One-Phase Mixture

Before exploring further the nature of lipid phase separations, another possibility should be discussed: A particular spatial organization in a lipid mixture might be described as a structured one-phase region instead of phase coexistence. In this state of matter, molecules are organized on the nm and tens of nm scale, but chemical potentials can vary with mixture

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gion, then we would expect to find a first-order
transition to a two-phase region somewhere in
composition space. Sharp transitions can be
biologically useful, for example, in amplifica-
tion of stimuli (Stadtman and Chock 1977).

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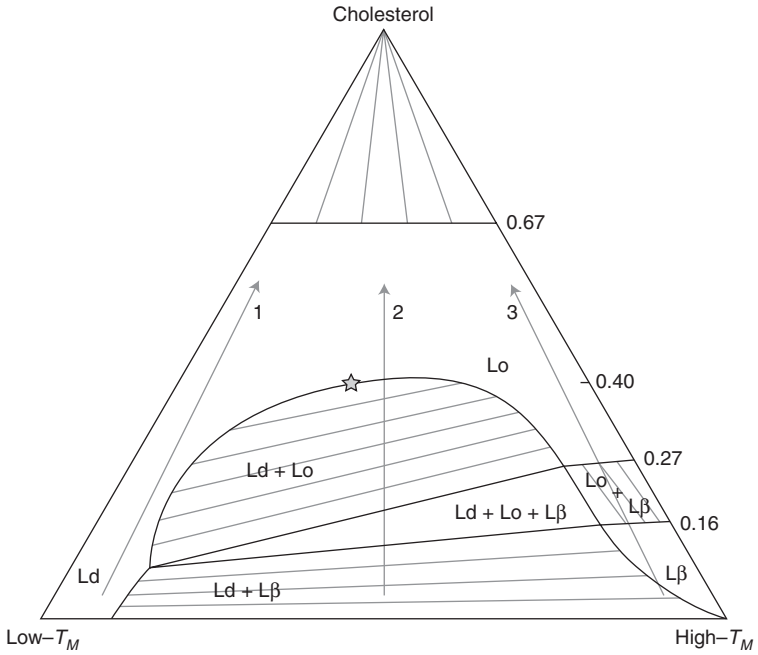

Figure 3. Illustrative phase diagram for a ternary lipid mixture containing low- and high-melting temperature lipids and cholesterol. Tielines are shown in phase-coexistence regions, and the Ld + Lo critical point is marked with a star. The effect of cholesterol addition to Ld (arrow 1), Lβ (arrow 3), or phase-separated Ld + Lβ mixtures (arrow 2) is discussed in the text.

increases in the Ld phase (arrow 1 in Fig. 3), apparently cholesterol continuously dissolves without phase separation right up to its maximum solubility of 67 mol %. The same is not true of the gel phase (arrow 3 in Fig. 3). In the Lβ phase, cholesterol reaches a maximum solubility of about 16 mol % (for SM, DPPC, or DSPC in mixtures with DOPC, POPC, or SOPC). Cholesterol chemical potential rises steeply at this special concentration, resulting in the formation of a Lo phase having the higher cholesterol concentration of ~27 mol %. The concentration of 16 mol % corresponds to each cholesterol molecule in the gel being surrounded by about six phospholipids—every phospholipid in the gel "solvating" but one cholesterol in the cholesterol-saturated solid Lβ phase. Cholesterol at concentration >16 mol % must be accommodated in a different type of lattice than that of the Lβ phase, namely that of the Lo phase. Apparently, lateral and rotational positions have larger ranges available for optimal cholesterol solvation when not dictated by the demands of the highly-ordered Lβ lattice. Perhaps the details of cholesterol molecular shape make anisotropic demands on its now approximately four or fewer phospholipid neighbors in Lo, rather than the six phospholipid neighbors of the cholesterol-saturated Lβ phase. Packing adjustments in Lo, unconstrained by the Lβ lattice, seem to enable each cholesterol to be shielded from water by fewer lipid headgroups.

As cholesterol concentration increases, eventually reaching its maximum solubility in the Lβ phase, the cholesterol also responds to the presence of any coexisting Ld phase. For example, Figure 3 arrow 2 corresponds to the addition of cholesterol to a two-phase mixture of Ld (DSPC-saturated DOPC) and Lβ (DOPC-saturated DSPC). In this interesting situation, the cholesterol has modest preference for Lβ over Ld, but dissolves into both phases. In Ld and Lβ, cholesterol chemical potential

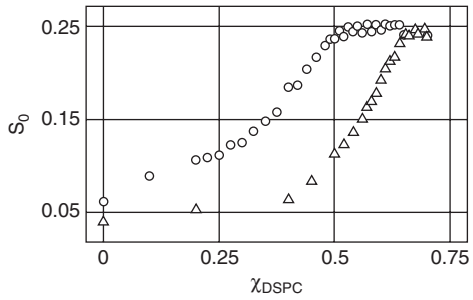

Figure 4. ESR reveals similarity of macroscopic and nanoscopic phase properties. Compositional trajectories run in the approximate direction of Ld + Lo tielines (see Fig. 3) and differ only in the identity of the low-T_M lipid. Composition-dependent order parameters obtained from ESR spectral simulations in DSPC/DOPC/chol (triangles) and DSPC/POPC/chol (circles).

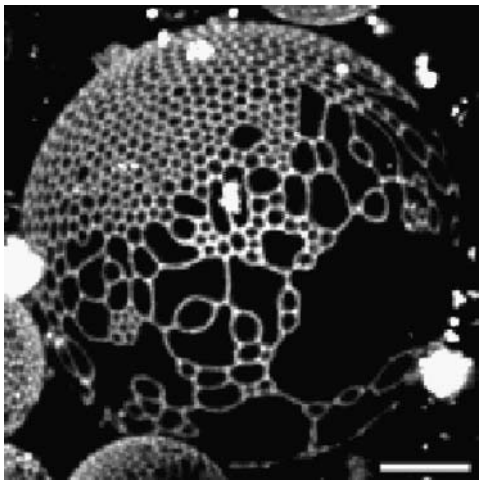

Figure 5. Confocal microscopy of giant unilamellar vesicles reveals interesting domain morphology in a four-component mixture that is intermediate in size between the smallest nanoscopic domains of DSPC/POPC/chol and the macroscopic domains of DSPC/DOPC/chol. Vesicle composition DSPC/DOPC/POPC/chol = 45/4.5/25.5/25. Scale bar 10 microns.

modulation of spatial organization that shows a number of possible morphologies, including the possibility of progression through different morphologies. For mixtures that show a range of size scales, for example the four-component mixture DSPC/DOPC/POPC/chol, the extremes of fluid phase morphology are known: micron-sized, round domains at 0 mol % POPC, and domains much smaller than 300 nm (and likely in the range of 2–5 nm) at 0 mol % DOPC. However, in the intermediate regime, different morphologies are frequently observed (Fig. 5). The systematic examination of such behavior in lipid-only models might inform a search for similar morphologies in cell membranes.

4. Are the domains observed with light microscopy strongly sensitive to intense illumination?

   For any imaging techniques in which the question is a transition from "close-to-phase-separated" to actually phase-separated, we expect sensitivity to light-induced macroscopic domains. This artifact can be minimized by use of low dye concentration, multiphoton excitation, and sensitive cameras (Morales-Penningston et al. 2010).

Value of a Phase Diagram for a Four-Component Bilayer Mixture

Because DSPC/DOPC/chol has macroscopic Ld + Lo phase separation, whereas DSPC/POPC/chol has nanometer-scale Ld + Lo separation, a minimal system with domain size controlled by composition would be the four-component mixtures DSPC/DOPC/POPC/chol. Finding the boundaries of phase regions in four-component mixtures will be data-intensive because a three-dimensional composition space must be examined. In addition to phase boundaries, domain size scales must be determined; GUV imaging and FRET are useful for obtaining such information. Domain lifetime distributions are necessary for understanding their role in the enzymatic activity of membrane proteins. Are there distinct protein behaviors in response to distinct lipid phase behaviors, or does a short domain lifetime effectively present the protein with an averaged environment?

CONCLUSIONS

It is clear that cell membranes possess domains with a range of sizes. Compositional heterogeneities also occur in lipid-only bilayer mixtures, with a range of sizes and perhaps other properties. Model mixtures in which domain size is controlled by composition from nanometer-scale to microns would provide a valuable experimental system for examining this aspect of membrane rafts. So far, no such systematic study has been published, though we have recently identified a four-component mixture that meets this criterion. Also of interest for the behavior of cell membranes, even single phase nonideal lipid mixtures can be induced to increase the size of their heterogeneities. Here, a case that plagues the use of fluorescence microscopy is the introduction of artifactual visible domains by intense illumination (Zhao et al. 2007b). This might be caused by free radical initiated polymerization that starts from the reactive excited singlet state (Ayuyan and Cohen 2006). More interesting examples come from aggregating ganglioside by binding cholera toxin B subunit, resulting in large domains (Hammond et al. 2005). Such controlled increase of domain size by protein binding or cross-linking could be used by cells in a controlled fashion to effect cellular functions (Lingwood et al. 2008). Furthermore, perhaps the cell has mechanisms for the complementary role of controlled decrease of domain size, for example, via protein "obstacles" that interfere with phase separation.

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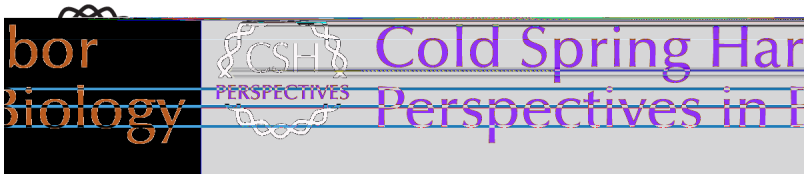

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