Guidelines to name and study plasma membrane domains in plants

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Abstract
Biological membranes play a crucial role in actively hosting, modulating, and coordinating a wide range of molecular events essential for cellular function. Membranes are organized into diverse domains giving rise to dynamic molecular patchworks. However, the very definition of membrane domains has been the subject of continuous debate. For example, in the plant field, membrane domains are often referred to as nanodomains, nanoclusters, microdomains, lipid rafts, membrane rafts, signaling platforms, foci, or liquid-ordered membranes without any clear rationale. In the context of plant-microbe interactions, microdomains have sometimes been used to refer to the large area at the plant-microbe interface. Some of these terms have partially overlapping meanings at best, while they are often used interchangeably in the literature. This situation generates much confusion and limits conceptual progress. Thus, there is the urgent need for us as a scientific community to resolve these semantic and conceptual controversies by defining an unambiguous nomenclature of membrane domains. In this perspective, experts in the field get together to provide explicit definitions of plasma membrane domains in plant systems and experimental guidelines for their study. We propose that plasma membrane domains should not be considered based on their size alone but rather according to the biological system being considered, such as the local membrane environment or the entire cell.

Plasma membranes are assemblies of billions of individual molecules arranged as an asymmetric lipid bilayer with integral and associated proteins. According to the fluid mosaic model of cell membranes initially proposed by Singer and Nicolson, biological membranes are "analogous to a two-dimensional oriented solution of integral proteins (or lipoproteins) in the viscous phospholipid bilayer solvent"[1]. As such, without additional constraints, proteins and lipids are predicted to diffuse within membranes, which would tend to homogenize over time. According to this theory, if one probes a continuous membrane at different 2D coordinates, the protein and lipid composition would be essentially similar. However, in vivo, there are several parameters limiting protein and lipid diffusion that counteract the homogenization tendency of viscous 2D structures[2]. This presents additional complexity and leads to the accumulation, or lateral segregation, of certain proteins and/or lipids in specific areas of an otherwise continuous membrane. As such, the fluid mosaic model has been updated many times to emphasize the mosaic character of membranes[3–5]. This heterogeneity is behind the very concept of **membrane domains**, which we define as the local accumulation of one or several membrane components (proteins and/or lipids) within specific areas of a membrane.

Two main plasma membrane organizational schemes.
Over the last few decades, the study of fundamental aspects of plant development, reproduction, interaction with microbes or response to abiotic stressors unveiled a common theme: the dynamic organization of the plasma membrane (PM) in various membrane domains. The blossoming of studies on PM domains in plants and other organisms has led to the proliferation of terminologies, creating some confusion. Here, we define and simplify the nomenclature of membrane domains.
The organization of the plasma membrane can be conceptually divided into two main organizational schemes which we propose to name **nanodomains** and **polar domains**. While polar domains and nanodomains present some similarities, and are likely interconnected, these membrane domains differ in the scale at which they are considered and in their functional purpose. Here and throughout this manuscript, the term "scale" does not refer to the size of the domains, but rather to differences in the biological system under study (e.g. difference between the molecular scale and the cellular scale). Indeed, the minimal unit for studying nanodomains

is a membrane plane and nanodomain formation is seen as a route to the local regulation of molecules and molecular complex function. By contrast, the minimal system to consider for a polar domain is the entire cell. Polar domains are functionally linked to the orchestration of cellular behavior such as the orientation of cell division and cellular growth[6,7]. They are accompanied by other hallmarks of cell polarity including polarized vesicular trafficking, localized cytoskeleton, and cell wall modifications. Each polar domain is typically found once or at a small readily defined number in a given plant cell. By contrast, nanodomains are usually found as repeated units within the same plane of the PM. Their number can reach up to hundreds of units within a membrane. Therefore, nanodomains may form the smallest discernible entity of the PM above the scale of single molecules. Polar domains represent regions of the PM where membrane constituents, including nanodomains, are organized at the cellular level in relation to a distinct polarity axis of the cell. In essence, we promote a concept in which polar domains are the result of cellular-scaled mechanisms (e.g., directed transport, oriented cytoskeleton), which are clearly distinct from domains arising from "local" mechanisms (e.g., protein-protein, protein-cell wall, protein-lipid, lipid-lipid interactions, or phase separation).

Nanodomains: molecular clusters within the PM plane

In a broad sense, we propose to define nanodomains as distinct PM environments that present local accumulations of specific biomolecules (Figure 1a, 1b). Thus, nanodomains are local macromolecular assemblies of proteins and lipids that are nanoscale in diameter (i.e., < 1 μm). In this context, nanodomains have also been referred to as nanoclusters, notably in studies that focused on specific lipid or protein species observed using single molecule imaging techniques[8–12]. Aside from the assumption that nanodomains are molecularly distinct from adjacent membrane region, the term nanodomain is exempt from any additional requirement on physicochemical properties such as overall composition, shape, oligomerization status, material properties, lifetime, function, or regulatory mechanisms.

Nanodomains are highly diverse. There are ample examples in the literature showing that there is not a single type of membrane nanodomain but that they are instead extremely diverse in nature[13–15]. In fact, biological membranes are made of a plethora of co-existing nanodomains with different compositions of proteins and lipids (Figure 1c). Given this diversity, it is critical to note that no single molecule (protein or lipid) or physicochemical property can be considered a universal landmark for all nanodomains. For example, it is often found in the scientific literature that REMORINs (REMs), FLOTILINs (FLOTs) and Hypersensitive-Induced Reaction (HIR) proteins are generic markers of nanodomains in plants[14,16,17]. Here, we would like to discourage this view in the future since many nanodomains at the PM of plant cells are component-specific and are not necessarily enriched in these proteins. Furthermore, different REMORIN, FLOTILIN and HIR isoforms localize to distinct nanodomains[13,18,19]. In practice, we propose the term nanodomain to be combined with the name of the biomolecule or property under investigation[20], e.g. "REM1.2 nanodomains". If there is an alternative functional word to refer to a specific type of nanodomain, we encourage using it, rather than the more generic nanodomain term (e.g. membrane contact sites[21]). Overall, nanodomains are relatively static or slow-moving structures in 2-D in the plane of the PM[22]. However, the dwell time of nanodomains at the PM can vary drastically. Some proteins or lipids can be very dynamic within nanodomains, with a short life-time (i.e., few seconds)[23], while others are persistent and can last for at least several minutes[10].

Nanodomain does not imply pre-defined physicochemical properties. The term nanodomain should not be used to define a single type of membrane composition or function, and nanodomains should not be assumed to be sphingolipid-, sterol- or phosphoinositide-rich. First,

technical constraints in cell biological imaging currently limit the holistic exploration of membrane organization. In particular, it is technically challenging to directly visualize a protein together with particular lipids in living plant cells. For example, there is not a direct proof that sterols specifically co-localize with certain nanodomain-organized proteins in planta. Second, while the assembly and/or the dynamics of certain nanodomains may depend on sterols, sphingolipids or phosphoinositides, this may not be true for all membrane nanodomains. Third, the way molecules assemble and exchange with their surrounding environment can significantly affect the dynamics of the molecules within the nanodomain itself. The behavior of a particular component in the nanodomain can further be influenced by the differences in biophysical properties between the nanodomain and its surroundings. Depending on the status of the macromolecular assembly, adding, or removing a component to or from the nanodomain or the surrounding environment can have varying effects on the behavior of other components with the same nanodomain. For example, external application of methyl-ß-cyclodextrin (MβCD), a cyclic oligosaccharide that depletes sterols from cellular membranes, has opposite effects on the dynamics and localization of FLOT1 and HIR1, two nanodomain-organized proteins[24]. Sphingolipids are thought to be mainly present in the outer membrane leaflet of the PM. Still, perturbing sphingolipids may affect lipid diffusion and the formation of certain nanodomains in the inner —cytosolic— leaflet[25]. Although they have not been described so far in plant membranes[25–27], compelling evidence for such coupling exists in animal cells. Furthermore, metabolic networks coordinating the abundance of different membrane lipid classes are largely unexplored in plants. Fourth, sterols are chemically and structurally diverse, with specific molecular species having different effects on membrane properties[28]. Fifth, distinct types of nanodomains – containing various sets of proteins – may be dependent on the same lipids (e.g., sterols, sphingolipids, or anionic lipids)[14,29]. Conversely, individual lipids may exhibit a dual distribution between nanodomains and diffuse localization pattern in the same cell[10,23,30].

It is also evident that nanodomain-organized molecules are not necessarily associated with specific lipid order (i.e., liquid-ordered membranes that comprise the liquid crystalline phase of the bilayer) or detergent-resistant membranes and those terms should never be used as synonymous to nanodomains. Likewise, it is crucial to make a clear distinction between nanodomains and lipid rafts. Lipid rafts were described in an iconic review by Kai Simons and Elina Ikonen in 1997 as dynamic membrane domains induced by the cooperative interactions of sphingolipids and cholesterol[31]. The very concept of lipid rafts, their dynamic nature, and their size have been the subject of much debate and controversy[2]. Despite its influence on our view of membrane structure, from today's perspective, the lipid raft concept falls short of fully encapsulating the spectrum of variability seen in nanodomains that can be found in cellular membranes. Furthermore, we discourage using "lipid raft" to describe the localization of plant proteins, since in practice, the evidence for nanoscale co-localization between a particular protein and sphingolipids/sterols at the nanoscale is lacking in plants.

Nanodomain-organized proteins are not necessarily in an active state. Molecules organized in nanodomains should not be assumed to be in an active state, a term that in biological systems is additionally difficult to define. While the stimulus-dependent organization of the small GTPase Rho-of-plants 6 (ROP6), REM1.2, Formin 6, or the receptor like-kinase LYSINE MOTIF KINASE3 (LYK3) in nanodomains has been linked to the activation of corresponding cellular or molecular events[10,32–35], recent reports suggest a more complex interplay between the organization of the PM and the functional status of its constituents. This can be illustrated by four prominent examples: 1) Both constitutively active and constitutively inactive forms of Arabidopsis ROP2 form nanodomains at the root hair initiation domain[36]. 2) The bacterial flagellin receptor FLS2 is organized in nanodomains in the absence of its cognate ligand[37–40].

3) *Solanum tuberosum* REM1.3 is organized in nanodomains without stimulation[41], while its active state correlates with a dispersed organization[42]. 4) The condensation of formin nanodomains by the bacterial effector protein XopR is associated with the sequential activation and inhibition of its actin nucleation activity[43].

Polar domains: PM domains at the cellular scale

In contrast to nanodomains, the term microdomains was previously proposed to define membrane domains above 1 µm in size[44]. However, the terms "microdomain" and "nanodomains" have been used synonymously for many years. Moreover, the term microdomain implies by historical definition an enrichment in sterols and sphingolipids[45–47], which is not supported by the current data. The formation of these PM domains is often the manifestation of cell polarity[6,7,48]. Thus, to avoid ambiguity, we propose using polar domain, a term coming from the field of plant cell polarity[6,49–55] (Figure 2a-f). Cell polarization refers to the process by which a cell establishes and maintains distinct regions with specific molecular composition leading to specialized structures and functions. In this context, polar domains can be defined relative to cell geometry, for instance, the different faces of the cell and its geometric edges[55]. The cell can also be polarized by specific mechanical or environmental cues[6]. For instance, the interfacial membrane (i.e. the host-derived membrane) established during particular host-microbe interaction is continuous with the PM but has a very specific biochemical composition and the plant-microbe interface constitutes a strong polarizing cue[29,44,56]. Similarly, polarized cells, such as root hairs or pollen tubes contain distinctive sub-apical polar PM domains with a highly specific lipid and protein composition[57]. Another example is the PM region where the future cell plate will fuse to the PM during cell division, referred to as the cortical division zone/actin depleted zone. It exhibits a distinct cytoskeletal organization and unique protein composition and may therefore be considered a polarized domain[58].

Polar domains are not necessarily found only once in the cell. For example, ROP11, found in differentiating xylem cells, accumulates in self-organized membrane domains that are several micrometers in size[59,60]. These ROP11-containing domains are akin to other polar domains in the sense that they lead to cellularly localized and polarized vesicular trafficking, cytoskeleton organization, and cell wall modification. The lobes and neck of pavement cells, which are labeled by ROP2 and ROP6, respectively, can also be seen as polar PM domains[6,61].

Nested organization of the plasma membrane.

Various polar domains and nanodomains co-exist within cells, conferring a nested organization to the PM (Figure 2a). Indeed, proteins or lipids are often organized in nanodomains within a polar domain (Figure 2e and 2f). This is the case of the lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in growing pollen tubes. Indeed, sensors for this lipid accumulate at the flank of the tip in growing pollen tubes, in a pattern representing a polar PM domain[57,62,63]. In addition, within this polar domain, a portion of PtdIns(4,5)P_2 sensors are organized in nanodomains[23]. During root hair initiation, the Rho GTPase ROP2 and its regulator (i.e., GTPase Exchange Factor (GEF3)) accumulate at the root hair initiation domain (RHID), which is a polar domain[54]. Within this domain, GEF3 and ROP2 are themselves organized in nanodomains[36]. The same is likely true for self-organized ROP domains that are clusters of ROP-containing nanodomains[64], or for ROP6 in the neck of pavement cells[11]. Another example is the auxin efflux carrier PIN2 found in nanodomains within a polar domain located on the apical (shootward) face of root epidermal cells[51]. In the context of plant-microbe interactions, REM1.3 is organized in nanodomains within the polar domains formed by the extra-haustorial membrane during the infection by *Phytophthora infestans*[65]. Of course, not all molecules in

polar domains have been described as organized in nanodomains (Figure 2d). For example, PtdIns(4,5)P_2 accumulates throughout the extra-haustorial membrane following powdery mildew infection[66] and at the tip of growing infection threads harboring symbiotic bacteria during the establishment of the root nodule symbiosis in legumes[56]. It should also be noted that in many cases, the nanoscale organization of polarly-localized molecules remains undefined and/or might be difficult to assess due to technical limitations in imaging three-dimensional membranes at the required resolution.

As is often the case in biology, many of the characteristics apparent at a given scale cannot be reduced to the properties of lower-scale components, although they are determined by the latter. Yet, it is worth noting that polar domains can emerge from the precise positioning of nanodomains. Perhaps the best-documented example is the formation of the Casparian strip domain in the root endodermis. Indeed, the Casparian strip membrane domain proteins (CASP) are initially found in small distinct nanodomains in differentiating endodermis cells[67,68]. As the cell differentiates, those CASP-enriched membrane nanodomains get positioned into bigger polar membrane domains that can reach several micrometers in size, until they eventually appear as a single continuous belt that divides the inner and outer faces of endodermal cells. Another example is the SOSEKI protein family. Some SOSEKI proteins highlight a polar domain in cell corners[69]. SOSEKIs are first localized in puncta, akin to a nanodomain organization, and polymerization of the SOSEKI proteins mediates the formation of a polar domain centered at the cell corner[70]. Hence, polar domains can be composed of nanodomains and the cellular positioning of nanodomains can lead to the formation of polar domains (Figure 2a-f). Finally, the formation and maintenance of a polar domain can be mediated by processes defined at the nanoscale within nanodomains, such as endocytosis or the formation of diffusion barriers.

Common concepts associated with the formation and maintenance of membrane domains.
Driver and client molecules of membrane domains. While the minimal definition of a membrane domain is the local accumulation of at least one type of molecule, it is likely that in reality membrane domains are much more complex entities (Figure 1c). In this context, we can differentiate "*driver*" molecules that are necessary for the formation of the membrane domains - and "*client*" molecules that are accumulating in the domain, but are dispensable for its formation and maintenance[71] (Figure 3a). In the case of the Casparian strip domain, CASPs and ESB1 would be drivers, and RBOHF or PER64 would be clients, as their loss-of-function does not affect the formation or integrity of the domain[72,73]. While such driver/client relationships have been explored to some extent for polar domains[71], they are far less understood in the case of the formation of nanodomains. We can expect that, as our knowledge on nanodomain expands, the question of client vs driver molecules for nanodomain formation and localization will become more prominent.

Self-organizing principles for membrane domain formation. The client/driver notion mentioned above is not trivial, since many membrane domains are likely formed and/or maintained by self-organization[35] (Figure 3b). Indeed, some molecules may not be required for initial symmetry breaking in the PM, but they may contribute to domain maintenance, size-regulation or stabilization. A classical example of a self-organized membrane domain is the polarized localization of ROP at the tip of root hairs or pollen tubes[57,60,74]. Indeed, ROPs interact with phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks), that synthesizes polar PtdIns(4,5)P_2[23]. This lipid in turn interacts with the ROP polycationic C-terminal tail thereby contributing to the polar localization of these small GTPases[60]. This serves as a conceptual

example featuring only a limited number of molecular components. However, in most cases, it is likely that a more extensive array of molecules, engaged in reciprocal feedback loops, play a role in membrane domain formation and regulation.

Border control. Another complex issue is what defines the frontiers between membrane domains. Membrane domains can be isolated from the rest of the PM by a diffusion barrier, or they may act as diffusion barriers themselves (Figure 3c and 3d). Such barriers limit or block the lateral diffusion of proteins and/or lipid molecules[25]. The barrier can be constituted of cytoskeleton components, transmembrane proteins, specific lipids influencing membrane diameter, modified cell wall materials, or a combination of those elements[25]. For instance, cortical microtubules limit the diffusion of ROP11 in differentiating xylem cells[75]. Membrane domains can also restrict the diffusion of external membrane components, which can be explained by their intrinsic properties, such as a specific electronegative signature, acyl chain saturation and length, membrane thickness or packing. One prominent example is the membrane diffusion barrier generated by the CASP domain, which limits the diffusion of endogenous membrane proteins and of membrane lipophilic dyes[25,76]. However, strict diffusion barriers do not always exist and membrane domains can be maintained via phase separation processes, macromolecular assemblies or polarized trafficking[12,14,51]. For example, localized sites of exocytosis and endocytosis can precisely define and position polar domains in various cell types, such as the root epidermis, protophloem, protoxylem, or pollen tubes[51,57,77–79].

Nanodomains can also be associated with phase separated cytosolic condensates (Figure 1c, subpanel iv). In such cases, the presence of the cytosolic membraneless condensates may influence the organization of membrane domains and/or modulate membrane curvature as observed for the tonoplast[80]. Reciprocally, membrane domains can nucleate liquid-liquid phase separation of cytosolic components[14,81,82]. However, a nanodomain does not necessarily correspond to or associate with a condensate. Furthermore, condensates can be cytosolic, and, in this case, cannot be defined as a membrane domain. Thus, these terms should not be used interchangeably. However, a phase-separated condensate, when associated with membrane, should be considered part of the membrane domain.

How to define whether a molecule is organized in a nanodomain?

Microscopy-based definition of membrane domains. Because the above-proposed definition is inherently based on the local enrichment of specific molecules, microscopy-based methods are the techniques of choice to study membrane domains. Local enrichment of compound within a membrane can be defined in fixed samples using electron microscopy coupled with immuno-gold labeling or by using live-cell fluorescence microscopy (e.g. confocal microscopy, variable-angle total internal reflection fluorescence microscopy (VA-TIRFM, Figure 4m-o), photoactivated localization microscopy (PALM, Figure 4p-r)). The latter is by far the most widely used technique to study the organization and dynamics of live membranes and access the localization of individual labeled molecules (Figure 4a-r). In fluorescence microscopy, nanodomains appear as fluorescent foci that are brighter than the rest of the membrane (Figure 4f-r). Depending on the density of these structures and the degree of accumulation, the observation of such organization can require high or super-resolution microscopy. Cellular membranes are not all equally accessible for high-resolution fluorescence imaging techniques, which is why most of the available data are representing the PM. In some cases, the molecule of interest is found in bright fluorescent foci, with no or little signal in the rest of the PM (Figure 4f-j). However, it is also possible that the protein or lipid is found both in brighter fluorescent foci and diffuse in the PM (Figure 4k and 4m-o)[10,23,83,30,32]. Plant membrane components can

be studied using correlative light-electron microscopy (CLEM)[81,84–86] in which a fluorescence signal is correlated with electron micrographs from the same sections. In combination with super-resolution microscopy[87,88], CLEM can provide information of the nanoscale organization of membrane components and of their native structural cellular context.

One weakness of using a microscopy-based operational definition of membrane domains is that classification is inherently dependent on how images are acquired (e.g., system resolution, or speed and sensitivity of acquisition), processed, or analyzed. Therefore, we advise not to rely on heavy image denoising or deconvolution methods to define nanodomain organization. Although machine-learning-based methods are evolving and improving rapidly, post-acquisition image analysis algorithms should be used with caution as they may create patterns and false-positive structures in a rather homogeneous fluorescence field. Furthermore, we advise that imaging and image processing and analysis conditions should be clearly stated and detailed. Local differences in PM image pixel intensity can be judged based on quantitative analysis of unprocessed images, with suitable statistical evaluation. In this regard, we advise to use automatized approaches on entire images rather than arbitrarily placed region of interest to avoid experimenter biases[23].

Biochemical, structural and computational characterization of membranes. Historically, the study of membrane heterogeneities started with biochemical purification, such as enrichment in detergent-resistant membranes (DRM)[89]. However, it is clear that a 2-phase purification approach cannot encompass the full range of membrane domain diversity. Thus, the presence or absence of a protein or a lipid in the DRM fraction should not be considered as a criterion for nanodomain organization. Along the same lines, sensitivity to lipid-perturbing agents (e.g., MβCD) or lipid-related mutants are not direct proof of nanodomain organization, nor for enrichment of specific lipids in the vicinity of the protein under study. For example, sterols have key functions in the overall organization of membranes and the intermolecular coherence between membrane lipids, and thus effects of modifying the PM sterol composition on observed nanodomain patterns may be indirect[90,91].

Some biochemical methods can also be helpful to complement microscopy approaches, as they are valuable techniques to characterize proteins and/or lipids enriched within a specific type of nanodomain. In particular, the rising use of proximity labeling techniques, with subsequent mass spectrometry, is a promising strategy to uncover the complexity of the nano-environment surrounding a protein of interest[92–95]. However, as yet, proximity labeling does not allow for sampling of the protein-associated lipid environment. Purification of membrane nanodiscs using detergent-free methods is an emerging way to simultaneously access the local proteome and lipidome associated with a molecule of interest, with the potential to enable biochemical characterization of a particular nano-environment[38]. The lipid nano-environment of proteins can also be accessed using soft ionization methods allowing mass spectrometry of molecular complexes and to infer the oligomerization state of proteins, lipid binding as well as the structural consequences of lipid–protein interactions[96,97]. Interactions between proteins and membrane lipids and protein-lipid structural interplay can also be analyzed by cryo-electron microscopy[98,99] (cryo-EM) or solid-state NMR studies[41,100,101]. Finally, complementary molecular dynamics (MD) simulations are powerful computational approaches, offering a spatially and temporally resolved, atomistic description of potential interactions between proteins and lipid molecules[102–104]. Combining MD simulations with highly accurate protein structure predictions obtained by methods based on machine learning[105] opens new possibilities to study membrane nano-organization. While these approaches can provide insights toward a mechanistic understanding of what may regulate the formation of a membrane domain, it shall

be noted that they do not constitute a basis to define whether a membrane molecule is organized in nanodomains in planta.

Concluding remarks

In this perspective, we aim to clarify the ambiguous use of alternative terms describing PM domains in plants to aid this active field of research. In principle, we divide membrane domains into two broad categories, nanodomains and polar domains, which represent two scales of membrane organization. We believe that this division will help avoid confusion and ambiguities, particularly between the terms nanodomains, microdomains and lipid rafts. We wish to emphasize that membrane nanodomains are diverse and should not be reduced to one particular subtype with a unique physico-chemical property, nor be assumed to be associated with an active state of its constituent components. With this in mind, the challenge for the coming years will be to define the biophysical features that regulate the dynamic lateral and transbilayer partitioning and organization of protein and lipid complexes, and to define the spatial and temporal logic underlying membrane-associated molecular events. These questions are far from understood in plants, which have several specific features likely impacting membrane dynamics and partitioning. Indeed, the cortical actin cytoskeleton has been widely described in animal cells as acting like a fence corralling the diffusion of membrane components[2]. However, it is still being determine whether a similar concept can be applied to plants, in which the structure and function of cortical actin are not as well defined. Cortical microtubules, another key attribute of plant cells, may also play a role as barriers for lateral diffusion within membranes. Furthermore, plants have a particular lipid composition, notably regarding sterols, sphingolipids, the plasticity of acyl chain length and saturation level of phospholipids[106–108]. Plants also have to remodel membranes in response to environmental and temperature variations (yearly and daily)[109]. The ensuing changes in acyl chain length and the saturation state of lipids will affect membrane fluidity and the lateral distribution of proteins with transmembrane regions. The plant cell wall is also known to impact the diffusion of membrane components, but how this is achieved at the molecular level is still largely unexplored[39,110,111]. Finally, we must define how mechanical connections and forces are actively modulated to execute and coordinate parallel and context-dependent molecular events within membranes[6,112]. With the progress of fluorescence microscopy techniques with ever-increasing spatiotemporal resolution, the study of membrane domains has become a major focus of plant cell biology, and we anticipate that this interest in the community will continue to grow in the coming years. Like Garth Nicolson[1,3–5], we should continue to improve and refine the fluid-mosaic model of membrane structure, considering the impact of plant-specific features on plasma membrane organization.

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Acknowledgements: We thank Christine Miège, and Niko Geldner for their help in preparing the manuscript. We thank Michelle von Arx (Fig. 4p, q and r) Vedrana Markovic (Fig 4g and h), Eva-Sophie Wallner (Fig.4e), David Biermann (Fig.4i, j and k), Andreas De Meyer (Fig. 4f), Maritza van Dop (Fig. 4d) and Liam Elliot (Fig. 4c) for providing the microscopy images shown in Figure 4.

Authors contributions: Project planning: Y.J., O.B., Y.B., M-C.C., A.M., S.M., F.S-P., E.Z., J.G., Writing of the draft manuscript: Y.J. and J.G., Edition and comments on the manuscript: Y.J., E.B., D.C.B., M.A.B., Y.B., T.O.B., M-C.C., G.G., I.H., P.A.H., C.K., A.M., Y.M., S.M., S.M., L.C.N., Y.O., T.O., X.P., R.P., M.P., S.R., C.S.R., F.S-P., E.R., D.V.D., J.V.N., D.W., S.Y., Z.Y., E.Z., J.G., Figure preparation J.G., Contribution to figure panels: Y.B. (Fig. 4a and b), L.C.N. (Fig. 4l), J.G.

Figure legends:

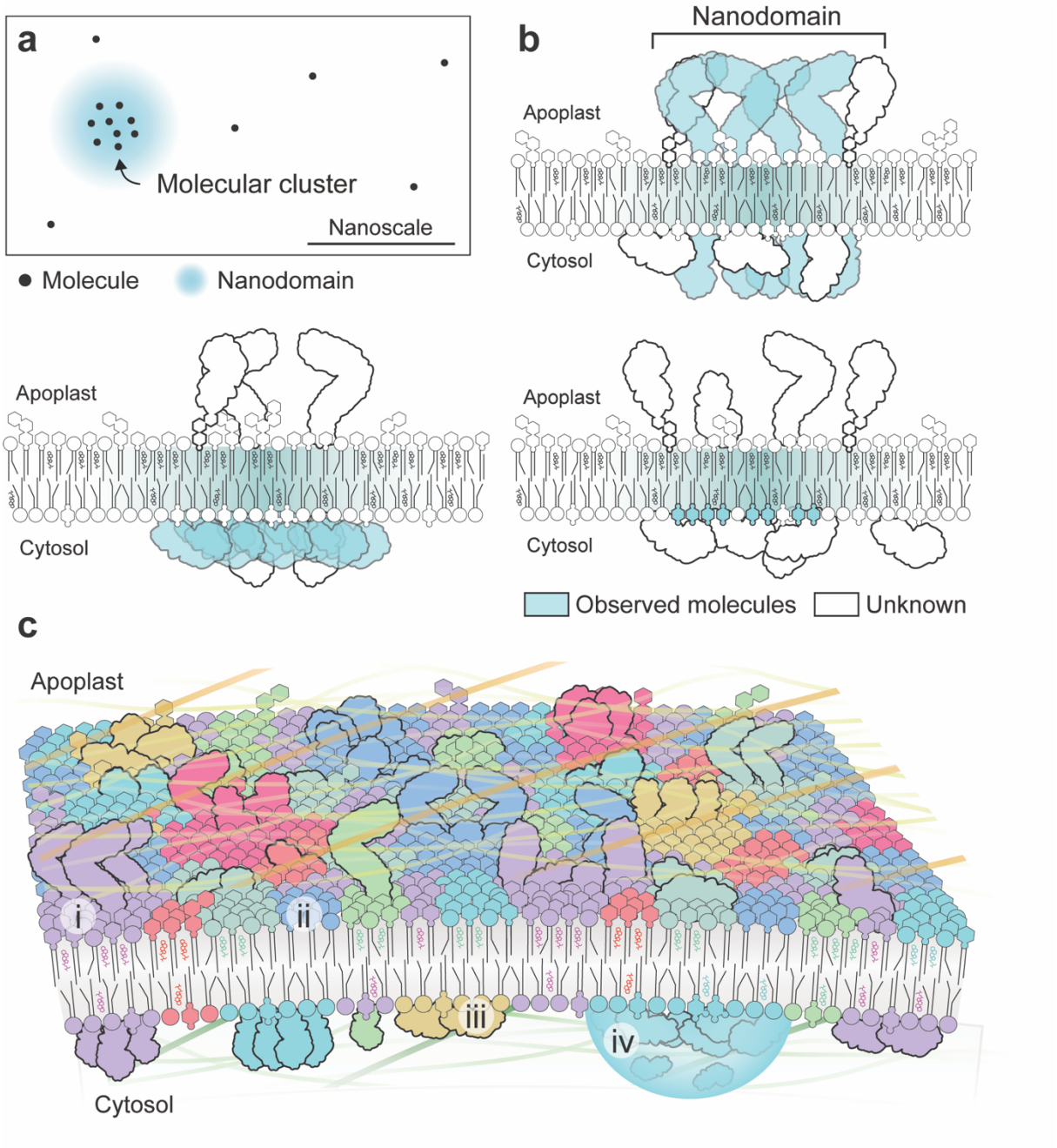

Figure 1: Plant plasma membrane nanodomains
a, Nanodomain refers to the local accumulation of molecules within a membrane plane, at the nanoscale. They are seen as molecular clusters using single molecule imaging techniques or can be observed as foci using confocal or VA-TIRF microscopy approaches. **b**, The properties, functions and overall composition of intrinsic proteins (top), peripheral proteins (bottom left), or lipids (bottom right) in nanodomains remain largely unknown. As an example, we represent here the molecules observed in blue and possible other molecular elements that remain to be identified, as unknown, in white. **c**, The PM is composed of a plethora of co-existing nanodomains, with different compositions of both proteins and lipids, represented here by distinct colors. Nanodomains can correspond to the assembly of molecules in both PM leaflets as in (i), or to the assembly of molecules in the outer (ii) or inner leaflet (iii). The PM can also act as a surface for the assembly of molecular condensates (iv) that can in turn regulate membrane lateral organization and curvature. These condensates, when associated with membranes, should be considered as part of the nanodomain.

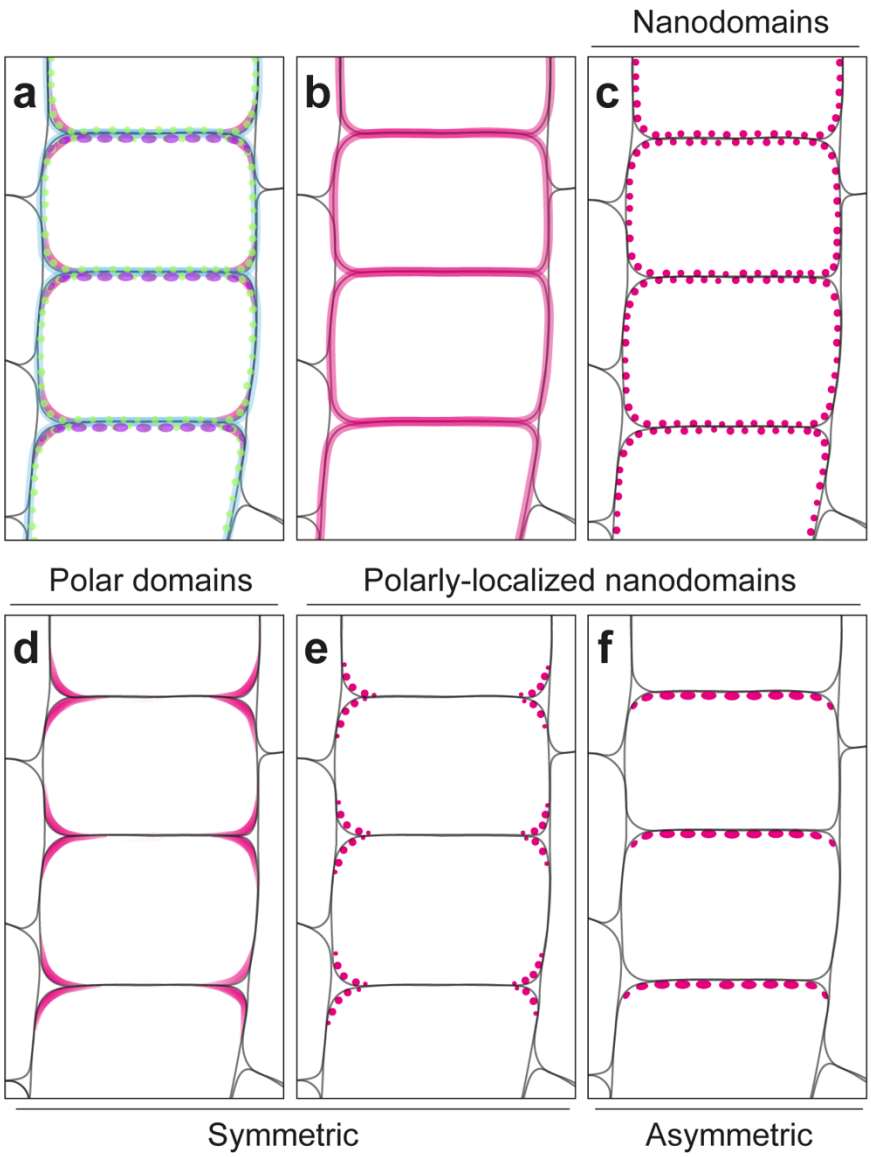

Figure 2: Nested organization of cell membranes into polar domains and nanodomains.
Cell membranes are dynamically organized into co-existing polar domains and nanodomains (**a**). Membrane constituents can be homogenously (**b**) or unevenly distributed (**c-f**) within a membrane. At the cellular level the localization of molecules is often set in relation to cell polarity which is established and modulated to regulate plant morphogenesis, development, reproduction and interaction with microbes. Nanodomains form the smallest discernible entity of a biological membrane above the scale of single molecules and consist, by definition, of both proteins and lipids. Polarly localized molecules can be – but do not necessarily have to be – organized in nanodomains. Conversely, nanodomains are not necessarily polarly-localized. For simplicity, nanodomains are here represented by dots whose size reflect protein accumulation at the cellular level and do not signify nanodomain sizes.

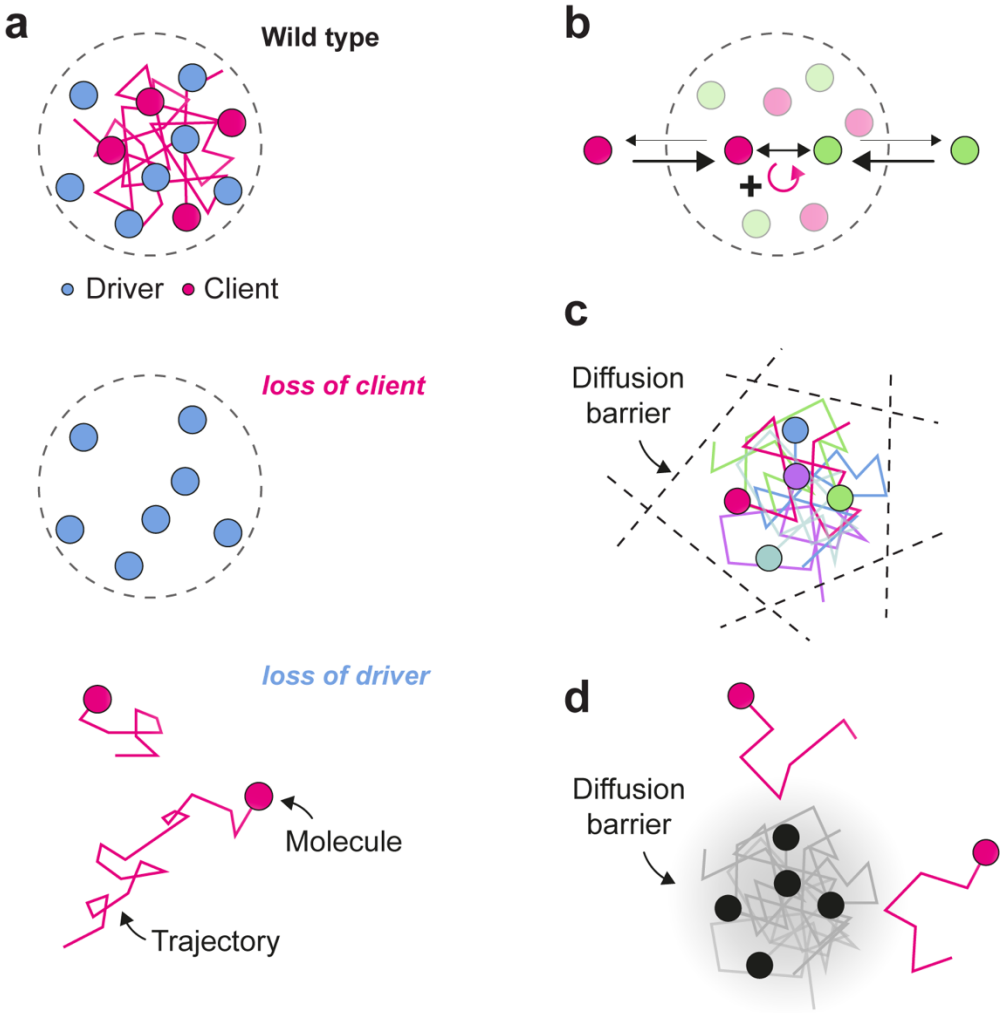

Figure 3: Emergence and maintenance of membrane domains
a, The formation of membrane domains can follow a "driver"- "client" relationship in which the loss of driver molecules impairs membrane domain integrity. Drivers can execute their function by binding physically to clients or by regulating membrane properties (e.g. curvature). Drivers can be integral membrane component as well as extrinsic to membranes as for example in case of membrane-associated phase separated cytosolic condensates. In the example in the middle, the absence of the "client" molecules does not impair the localization of the "driver" molecules, while in the example at the bottom, the "client" molecules fail to organize in nanodomains in the absence of the "driver" molecules.
b, Membrane domains can emerge and be maintained following self-organizing principles. In such a scenario, the formation of nanodomains results from the collective interactions of its individual molecules. Here, several elements promote the formation of a membrane entity and as such a potential "driver"-"client" relationship is not prevalent.
c, Membrane domains can be isolated from the rest of the membrane by a diffusion barrier. The barrier can be constituted of cytoskeleton components, transmembrane proteins, specific lipids, modified cell wall materials or a combination of those elements.
d, Membrane domains can constitute diffusion barriers for other molecules thereby maintaining membrane domain identity.
The examples given in a, b, c and d are not mutually exclusive and can cooperatively define membrane domain identity and integrity. Note that these principles can apply for the formation of both nanodomains and polar domains.

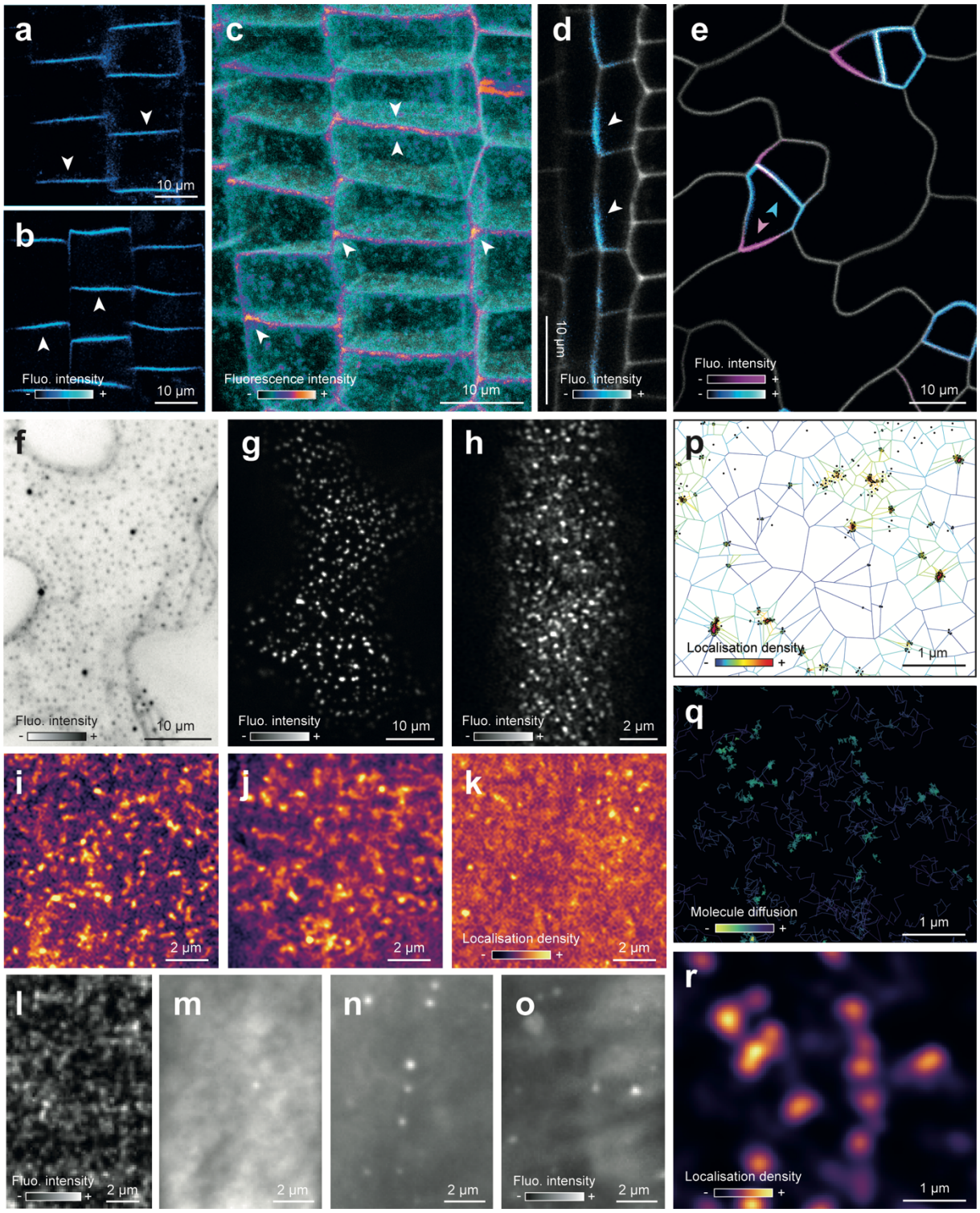

Figure 4: Microscopic observations of polar domains or nanodomains in the PM of plan cells
a-e, examples of polar domains observed by confocal microscopy. Localization of the auxin transporters, PIN1 (**a**), and PIN2 (**b**), at the rootward or shootward plan of root epidermal cells. In the same cell file, the cell surface receptor RECEPTOR-LIKE PROTEIN 4[113] marks the cell edges and corners (**c**). The protein SOSEKI 2 marks the inner basal edge of endodermal cells[69] (**d**). In cotyledons, BREVIS RADIX LIKE 2-YFP (shown in magenta) and OCTOPUS-LIKE 2 (shown in cyan) mark opposite polar domains in stomata and stomatal precursor cells[94,114] (**e**). Nanodomain organization of plasma membrane-localized proteins observed using different imaging modalities (**f-r**). Nanodomains can be seen as foci that that are brighter than the rest of the membrane (**f-o**). **f**, correspond to a maximum projection of confocal z-sections of *N. benthamiana* epidermis showing plasma membrane-associated molecular condensates formed by the

TPLATE complex subunit EH1deltaIDR3-mGFP[81]. Plasma membrane organization of the membrane contact site component GFP-NET3c[21] transiently expressed in *N. benthamiana* (**g**), or of the component of the endocytic machinery DYNAMIN-RELATED PROTEIN 1E (GFP-DRP1E)[115] in *Arabidopsis thaliana* root epidermis (**h**). **i**-**k** correspond to enhanced super-resolution radial fluctuations (eSRRF)[116] images of FLAGELLIN-SENSING2-GFP (**i**), BRASSINOSTEROID-INSENSITIVE 1-GFP (**j**) and FERONIA-GFP (**k**) in *Arabidopsis thaliana* cotyledon epidermis[19,37]. **l**, Confocal spinning disk image of the cellulose synthase complex subunit YFP-CESA6 in *Arabidopsis thaliana* root epidermis. **m**-**n**, VA-TIRFM images of ROP6-GFP in *Arabidopsis thaliana* root epidermis in control condition (**m**), upon auxin treatment (**n**) and osmotic stress (**o**)[10,30,32]. Nanodomains are seen as molecular clusters as observed by photoactivated localization microscopy (PALM) (**p**). **p** shows single molecule localization observed by PALM and segmented using Voronoï tessellation[117]. Black dots indicate the localization of single molecule. The Voronoï diagram (mesh) is color-coded based on the density of localizations and computed clusters are outlined in black. **q**, Observation of molecular clusters by single-particle photoactivated localization microscopy (spt-PALM), here clusters are seen as several molecules confined within the same nano-environment using Nanoscale spatiotemporal indexing clustering (NASTIC)[118] (**q**). Single molecule trajectories are color-coded based on their instantaneous diffusion coefficient. **r** depicts a 2D Kernel density estimation of single molecule localization of the same region (**q**), brighter foci correspond to region with higher density of localization.

Box1
Guidelines

- No assumption should be made on the composition, properties or function of a membrane domain. For instance, nanodomains are not necessarily active sites of signaling nor are they necessarily enriched in sterols and sphingolipids.

- The terms domain, polar domain or nanodomain should be combined with the name of the biomolecule or properties under investigation (*e.g.* REM1.2 nanodomains).

- The term nanodomain-organized is used to describe lateral organization of molecules in domains. The term nanodomain-localized implies that molecules associate with or localize within pre-existing membrane entities or compartments. Without experimental information suggesting the location of molecule to pre-existing domains, the terminology nanodomain-organized should be used to describe molecular clusters. Note that molecules that do not form clusters may locate in, or be associated, with membrane domains.

- We encourage the use of alternative and established functional terms to describe specific types of nanodomains (e.g. membrane contact sites, clathrin-coated pits) and polar domains (e.g. extra-haustorial membranes, CASP microdomain).

Box 2
Definitions:

Nanodomains: Nanoscopic (< 1 μm) membrane environment presenting a local accumulation of molecules, lipids and/or proteins, forming molecular assemblies within a membrane plane. Nanodomains are not singular structures but are repeatedly observed within a single membrane plane.

Nanoclusters: Molecules observed by single molecule localization microscopy approaches to be organized in clusters (in a way that quantitatively deviates from randomness). The observation of such cluster likely implies, but does not demonstrate, the co-occurrence, and/or co-clustering, of additional molecular elements that alltogether form nanodomains.

Polar domain: Site-specific accumulation of membrane molecules at the cellular level. Polar domains are usually asymmetrically distributed within a cell. Yet they can also be symmetrical when they define a cellular

polarity axis, for example by being localized at the cell equator. They are present once or in a small, easily defined, number of times in each cell.

Lipid order: Membrane lipid order is a biophysical parameter that defines a membrane organization and is often described by the degree of lipid packing. High packing corresponds to the liquid-ordered phase of the membrane (see below) and low packing to the liquid-disordered phase of the membrane.

Liquid-ordered: Describe the liquid crystalline biophysical state of membranes composed of tightly packed molecules and characterized by slow molecular diffusion. Lipid acyl chain saturation, sphingolipid hydroxylation and sterols composition are seen as predominant factors of membrane order level. Note that the use of tensiometric probes or membrane-order probes *in vivo* provides information about membrane properties which are not solely influence by lipids. Further, it should be noted that these probes are often pH sensitive.

Detergent-resistant membrane: Biochemical fraction obtained upon cold solubilization of membranes by the use of non-ionic detergents at a defined but arbitrary concentration.

Lipid rafts: Described as liquid ordered membrane domain whose formation is based on the preferential interaction between sphingolipids and sterols. Lipid rafts are proposed to nucleate the formation of proteo-lipidic membrane domains and selectively recruits membrane proteins.

Membrane domain driver: Proteins or lipids that are essential for or contribute to the establishment and/or maintenance of a membrane domain. They can also be described as membrane domain organizers or stabilizer.

Membrane domain client: Proteins or lipids, that are not essential for the formation and/or maintenance of a membrane domain but localize to a pre-existing membrane domain. They can also be described as effectors of membrane domains.

Phase: Corresponds to a homogeneous, physically distinct and mechanically separable portion of a system that has uniform properties.

Phase separation: Refers to the separation of molecules into two distinct phases, with different densities, compositions or properties. Phase separation is driven by the intrinsic properties of the molecules and/or by entropy and can be influenced by factors such as concentration, temperature, and molecular interactions.

Biomolecular condensate: Class of membraneless organelles that form via liquid-liquid phase separation processes. Condensates can be found at various subcellular localizations, and they can nucleate in the cytoplasm or nucleoplasm, but also at membrane surfaces. Membrane-associated condensates can form or be part of nanodomains or polar domains.

Diffusion barrier: Structures that prevent the lateral diffusion of membrane constituents. The cell wall, cortical cytoskeleton as well as transmembrane proteins and in some cases, lipids are suspected to form diffusion barriers.

Self-organization: Self-organization is the spontaneous emergence of a coherent and structured pattern (here membrane domains) within a specific system. This pattern arises as a consequence of the collective interactions of its individual molecules. These interactions are typically governed by simple rules, and through iterative feedback loops, they give rise to complex and organized structures.

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