ACS chemical biology

Reviews

pubs.acs.org/acschemicalbiology

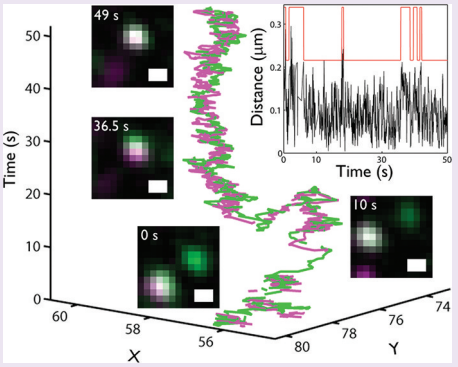
Nanoscale Membrane Organization: Where Biochemistry Meets Advanced Microscopy

Alessandra Cambi[†,*] and Diane S. Lidke[‡,*]

[†]Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

[‡]Department of Pathology and Cancer Research and Treatment Center, University of New Mexico, Albuquerque, New Mexico, United States

ABSTRACT: Understanding the molecular mechanisms that shape an effective cellular response is a fundamental question in biology. Biochemical measurements have revealed critical information about the order of protein−protein interactions along signaling cascades but lack the resolution to determine kinetics and localization of interactions on the plasma membrane. Furthermore, the local membrane environment influences membrane receptor distributions and dynamics, which in turn influences signal transduction. To measure dynamic protein interactions and elucidate the consequences of membrane architecture interplay, direct measurements at high spatiotemporal resolution are needed. In this review, we discuss the biochemical principles regulating membrane nanodomain formation and protein function, ranging from the lipid nanoenvironment to the cortical cytoskeleton. We also discuss recent advances in fluorescence microscopy that are making it possible to quantify protein organization and biochemical events at the nanoscale in the living cell membrane.



In 1972, Singer and Nicholson proposed the Fluid Mosaic model, in which most membrane constituents diffuse rapidly and randomly about the two-dimensional surface of the lipid bilayer.[1] However, live cell imaging techniques such as single particle tracking have provided considerable evidence that many receptors and even lipids are restricted in lateral mobility. These observations, along with biochemical techniques, established a compartmentalized view of the plasma membrane, which focuses around three hypotheses of microdomain organization: lipid rafts,[2] protein islands,[3] and actin corrals.[4] What remains to be understood is the specific contribution of these microdomains in regulating the signaling process.

There is mounting evidence for critical roles of the lipid nanoenvironment in regulating protein interactions. Favored interactions between certain types of lipids lead to their co-segregation in domains at the cell membrane, which led to the lipid raft theory. However, recent evidence is demonstrating that membrane organization is more complex than simple division of raft and nonraft regions. Also, proteins associated with the plasma membrane often undergo a lipid-based post-translational modification with the addition of an acyl chain to specific amino acids that can subsequently mediate the interaction of this protein with the lipids of the plasma membrane. Therefore, to fully characterize protein−protein interactions and understand the critical roles of lipids and membrane organization in regulating those interactions, it is important to study signaling events in living cells at high temporal and spatial resolution.

BIOCHEMICAL PRINCIPLES REGULATING MEMBRANE NANODOMAIN FORMATION

The formation of membrane nanodomains originates from lipid−lipid, lipid−protein, and protein−protein based interactions, which implies the existence of a variety of biochemical principles that allow these interactions to occur at the molecular level. The major structural lipids in eukaryotic membranes are the glycerophospholipids that share a similar hydrophobic portion but have different polar headgroups that confer a specific molecular geometry to each phospholipid, thus contributing to the regulation of membrane curvature. The other class of polar structural lipids is the sphingolipids. They contain two saturated hydrophobic chains that are longer and narrower than the phospholipids, pack tightly, and confer rigidity to the lipid bilayer. The sphingolipids straight chains and headgroup spacing favor the intercalation of cholesterol, which further contributes to increasing the lipid packing density.[5] These strong interactions between cholesterol and sphingolipids promote their co-segregation in domains at the plasma membrane, generally termed "rafts". Lipid rafts can sequester specific signaling proteins and allow the formation of supramolecular signaling complexes.[6] Proteins that reside within cellular membranes have molecular features that allow them to be embedded in the highly hydrophobic milieu of the lipid membrane. For some membrane-spanning proteins, the

Received: August 29, 2011
Accepted: October 17, 2011
Published: October 17, 2011

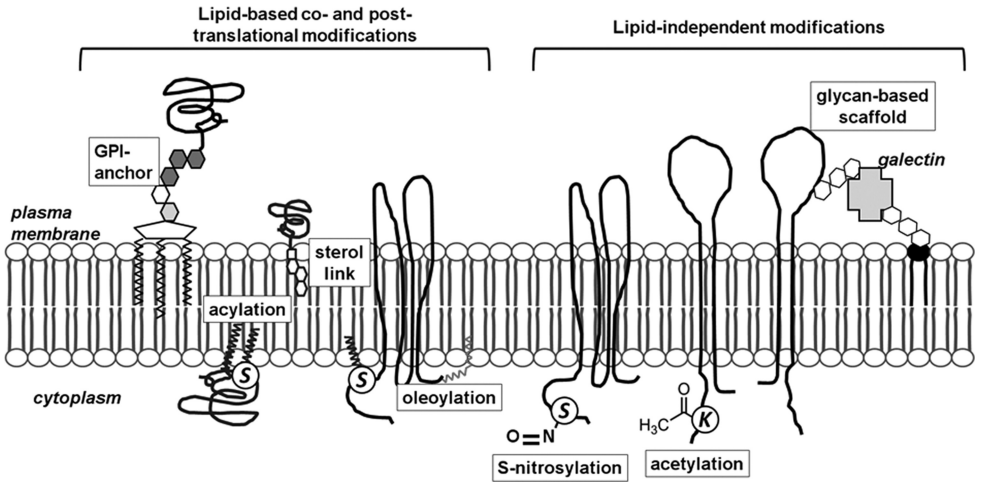


Figure 1. Biochemical principles regulating partitioning and nanoscale organization of membrane proteins. The formation of membrane nanodomains originating from lipid−lipid, lipid−protein, and protein−protein based interactions implies the existence of a variety of biochemical principles that allow these interactions to occur at the molecular level. Proteins associated with cellular membranes have molecular determinants that allow them to be embedded in the highly hydrophobic milieu of the lipid bilayer. Several forms of lipid-based modifications provide the proteins either permanently or transiently with the right membrane anchor. Non-lipid modifications further contribute to the fine-tuning of receptor function and subsequent signal transduction. The same protein can undergo different modifications; however, the regulation and interplay of these modifications are still unknown.

transmembrane domains typically consist of α-helices or β-sheets with their hydrophobic amino acid residues interfacing the hydrocarbon chains of the lipid bilayer. Alternatively, the association of proteins with the membrane can be mediated by specific co- or post-translational additions of lipid anchors such as the glycophosphatidylinositol (GPI) anchor, myristic acid tail, palmitic acid tail, *etc.* (Figure 1). Furthermore, membrane proteins often bear other non-lipid post-translational modifications (*e.g.*, glycosylation, S-nitrosylation) that might mediate the interaction with specific signaling components or scaffold molecules, thus contributing to the formation of functional membrane nanocompartments. The role of these non-lipid modifications in the organization of membrane nanodomains is still largely unexplored.

Lipid Nanoenvironment. Although the term "rafts" remains controversial, the existence of lipid and protein nanodomains at the cell membrane is now widely accepted.[7,8] It should be noted that while liquid-ordered domains readily assemble in artificial membranes, their existence in complex cellular membrane preparations has only recently been observed,[9] and direct detection in intact cells has proven more challenging. However, non-overlapping nanodomains of the glycosphingolipids GM1 and GM3 as well as spatially distinct sphingomyelin (SM) rich clusters have been detected,[10,11] highlighting the compartmentalized nature of the plasma membrane.

In many forms of cell activation, including malignant transformation or pathogen invasion, the enzyme acid sphingomyelinase (SMase) hydrolyzes SM into ceramide, which is released within the cell membrane and alters its biophysical characteristics.[12,13] Therefore, changes in plasma membrane sphingolipid levels are likely to affect the function of signaling molecular complexes by altering the lipid nanoenvironment. Extensive atom-scale simulations of ternary raft mixtures containing cholesterol, sphingomyelin, and phosphatidylcholine have shown nanoscale lateral heterogeneity and lateral pressure profiles clearly distinct from nonraft mixtures. Changes in lipid content might modify the lateral pressure profile, in turn altering the function of certain classes of membrane proteins.[14] Recent studies demonstrated that a voltage-gated potassium channel and its surrounding membrane lipids together represent a functional unit since the annular lipids were able to control the channel conformational switch from activated to resting state.[15] Similarly, Coskun and colleagues demonstrated that the membrane lipid composition does not alter the ligand binding properties of the epidermal growth factor receptor (EGFR) but rather modulates its allosteric structural transition from inactive to a signaling competent dimer.[16]

Another important lipid component of the plasma membrane is cholesterol, which is known to play an essential role in regulating the biophysical properties of membrane proteins and lipids. By sequestering the downstream effector partners but not the β2-adrenergic receptor in lipid-ordered domains, cholesterol regulates the nanoscale organization of the receptor signaling machinery.[17] Interestingly, cholesterol has been shown to induce a tilt in glycolipid headgroups, ultimately modulating glycolipid-dependent surface recognition processes such as presentation of erythrocyte blood groups or sperm activation.[18] Changes in cholesterol content at the cell membrane could therefore modulate glycolipid conformation by either masking or unveiling specific glycolipid sites, which in turn might affect interactions with other membrane molecules both in *trans* and in *cis*. Cholesterol is a major contributor to membrane fluidity. It has been shown that cholesterol coalesces with mobile FcεRI upon synapse formation but avoids immobilized receptors, suggesting that membrane constituents are attracted to cholesterol-rich regions due to fluidity.[19] Finally, cholesterol appears to be the glue that mediates raft-based interconnectivity at the nanoscale that might constitute the basis for large-scale raft coalescence observed upon cell activation.[20]

Lipid rafts of variable biophysical properties and molecular composition have been found in plasma membrane preparations, indicating that biological membranes have the capacity to form lipid nanoenvironments of continuously variable size, composition, and stability that represent the basic organizing principle of membrane compartmentalization.[21] The formation

dx.doi.org/10.1021/cb200326g|*ACS Chem. Biol.* 2012, 7, 139−149

of liquid-ordered domains at the plasma membrane rich in cholesterol and glycosphingolipids represents the dominant theory to explain raft existence.[5] However, it is unlikely that the liquid-ordered domains alone give rise to the great variation in lipid and protein content exhibited by rafts. Recently, a "lipid matrix model" of raft structure has been proposed that provides a plausible mechanism to explain how membrane rafts can achieve such a molecular complexity.[22] The lipid matrix model takes into account the putative role of asymmetric sphingolipids (*i.e.*, sphingolipids bearing *N*-acyl fatty acid tails of different length) in raft formation and function and envisages the existence of quasi-crystalline domains. It has been proposed that the liquid-ordered domains, formed by symmetric sphingolipids and cholesterol, may function as a matrix for recruiting raft proteins, including transmembrane proteins that stably reside at the cell membrane and are connected to the cytoskeleton. In addition, quasi-crystalline domains formed by asymmetric sphingolipids and phospholipids represent a matrix into which proteins tethered to the raft *via* GPI anchors or acyl chains can assemble and cluster. Additional interactions between the carbohydrate moiety of the glycolipids and the neighboring proteins adds yet another level of specificity that contributes to raft diversity.[22]

Whereas increasing information is available about the lipids in the plasma membrane outer leaflet, the composition, organization, and function of the inner leaflet are less well addressed. Phosphoinositides (PIPs) are concentrated at the cytosolic surface of membranes and become reversibly phosphorylated by PI-kinases. Differentially phosphorylated PIPs display unique subcellular distribution with preferential localization to subsets of membranes.[23] PIPs contribute to the unique negative charge of the inner leaflet, the bilayer asymmetry, and importantly, the differential targeting and trafficking of signaling proteins to the plasma membrane.[24,25] A recent elegant biophysical study by Lasserre *et al.* demonstrated the existence of highly dynamic lipid nanodomains in both the outer and the inner leaflets of the plasma membrane of T lymphocytes and the negative effect of raft alteration on the PI3-kinase pathway.[26] It remains to be established how receptors interacting with classical raft domains at the outer leaflet can signal across the membrane leaflet to these PIPs-enriched signaling domains.

The notion of lipid nanodomains with different molecular composition and physicochemical properties represents a clear advancement of our understanding of lipid rafts from undefined, elusive lipid platforms to fundamental biochemical entities responsible for membrane compartmentalization specificity.

Biochemical Modifications of Membrane Proteins. The lipid and protein composition of many membrane domains has been extensively investigated. Despite this, the underlying biochemical principles that determine how such a great variety of proteins associate with the plasma membrane and partition into specific nanodomains are still not entirely understood. To allow their embedding in the hydrophobic environment of the plasma membrane, proteins need dedicated moieties that can originate directly from their own amino acid sequence or from co- and post-translational modifications (Figure 1).

The most common lipid-based modifications found in membrane associated proteins are (i) the addition of GPI anchors, (ii) cysteine acylation also known as palmitoylation, (iii) prenylation and myristoylation, and (iv) the addition of sterol moieties at the C-terminus.[27] While GPI anchors[28] and palmitoylation[29] are known to mediate protein partitioning into the tightly packed lipid rafts, prenylation[30] and myristoylation[31] seem to target proteins to less restrictive nonraft membrane areas. Furthermore, the addition of a GPI anchor, prenylation, and myristoylation are stable cotranslational modifications, whereas palmitoylation is dynamically regulated by enzymes and is therefore reversible.[32] A typical example is the different raft affinity of the Ras protein isoforms that is dictated by the type of lipid anchor: while the doubly palmitoylated H-Ras strongly resides in lipid rafts, the prenylated K-Ras does not partition into lipid rafts, despite their significant homology in amino acid sequence.[12,33] Similarly, differential fatty acylation of membrane proteins has been shown to play an important role in T cell signaling, where localization and functional state of key signaling molecules, such as Lck and Fyn, and of coreceptors and adaptors involved in T cell activation have been shown to be regulated by the presence of specific lipid chains.[34]

In addition to the unique sterol modification of the Hedgehog family proteins reported by Porter *et al.* in 1996,[35] a novel form of fatty acid acylation has been recently documented for the lens integral membrane protein Aquaporin-0.[36] Combining direct tissue profiling by mass spectrometry of lens sections with proteomic analysis, Schey and colleagues observed that N- and C-terminus of Aquaporin-0 were modified by palmitoylation and oleoylation, respectively. Oleoylation represents the addition of oleic acid to a lysine residue *via* an amide linkage and appears to mediate the localization of Aquaporin-0 into lipid raft fractions.[36] Future investigation may reveal further novel forms of protein modifications by fatty acid chains that direct membrane targeting and nanodomain partitioning.

A significant number of recent studies have focused on lipid-independent reversible redox modifications of specific cysteine residues as a new cell signaling mechanism.[37] Nitric oxide produced from L-arginine by nitric oxide synthase enzymes can directly modify cysteines by covalent attachment resulting in the so-called S-nitrosylation. Important signaling molecules such as Ras,[38,39] β-catenin,[40] and MyD88[41] as well as a number of G protein-couple receptors have been found to be regulated by S-nitrosylation. Both acting on the cysteine residues, S-nitrosylation and palmitoylation are likely to have a dynamic interplay that could further fine-tune membrane receptor organization and cell signaling events. In addition, the nitric oxide has been shown to displace palmitate from proteins.[42] The development of more sensitive techniques to monitor nitrosylation will most likely reveal novel molecular dynamics controlling the recruitment of proteins to the plasma membrane and modulating signal transduction.[43]

A steadily growing number of studies indicate acetylation of non-histone proteins as another important lipid-independent post-translational modification that can modulate multiple cellular processes from gene expression to receptor activity.[44] Acetylation is the transfer of an acetyl group from acetyl-CoA to the e-NH_2 group of the side chain of lysine residues by a lysine acetyltransferase. Predominantly known for its role in the regulation of gene expression, lysine acetylation is now recognized as an essential player in the regulation of cell activities such as cytoskeleton organization, cellular transport, and protein stability.[45] Interestingly, the involvement of acetylation in the regulation of membrane receptor function is also emerging. Acetylated tubulin has been shown to specifically interact with the cytoplasmic domain of the membrane-associated sodium pump Na+/K+-ATPase, which therefore acts as microtubule-membrane anchorage site. Furthermore, at

the plasma membrane the prolactin receptor has been found to undergo cytoplasmic loop dimerization that depends on acetylation of multiple lysine sites along the loop and is essential to initiate the downstream signaling cascade.[46]

All of these competing reversible posttranslational modifications must be regulated by a complex interplay among different modifying enzymes and contribute to the dynamic regulation of nanoscale organization and function of a variety of membrane receptors. Together with the stable cotranslational alterations, these modifications represent additional tools used by the cell to fine-tune signal transduction.

Role of Glycans in Membrane Compartmentalization. In eukaryotic cells, glycosylation is a widespread posttranslational modification of secreted and membrane-anchored proteins as well as proteoglycans and glycolipids. Galectins, a family of galactose-specific animal lectins, bind and cross-link branches of specific N-glycans present on glycosylated molecules at the cell surface.[47] In this way, galectins act as molecular organizers of the cell surface able to recruit proteins and lipids to compartments where homo- and heterotypic clustering can occur. This generates the so-called galectin scaffolds or lattices, the dynamics and composition of which are still unknown.[48]

Recruitment of proteins to galectin lattices has been shown to prevent receptors from uncontrolled clustering and signaling.[49] In fact, biophysical approaches such as FRET have allowed the visualization of galectin-3 oligomerization, suggesting that this protein is indeed able to form small aggregates where certain receptors could be recruited.[50] For example, the interaction of the T cell receptor with galectin-3 has been shown to negatively regulate T cell receptor response to antigens.[51] Conversely, galectin lattice promotes EGFR signaling by sequestering the receptor away from negative regulatory caveolin-1.[52] By binding to glycans present on raft associated glycolipids, galectins likely play an important role in regulating the communication between different types of membrane nanocompartments. Understanding the cross-talk between galectin lattices, lipid rafts and other types of membrane compartments in the regulation of receptor signaling represents one of the future challenges in membrane biology.

IMAGING MEMBRANE COMPARTMENTALIZATION

The biochemical events described above are responsible for dynamic molecular interactions that determine anchoring and detaching of proteins from scaffolds, the cytoskeleton, or other membrane proteins/lipids. This suggests that partitioning of membrane proteins is not a passive event, but rather the restrictions placed on protein diffusion and localization regulate receptor accessibility to interaction partners, subsequently regulating signaling events. Therefore, understanding the contributions of microdomains to receptor function is needed to fully understand how signaling is regulated. Fluorescence imaging techniques (see Table 1) are providing new insights into membrane organization.

The Elusive Lipid Rafts. As discussed above, the lipid nanoenvironment is considered to have a critical influence on cellular function. Despite the biochemical evidence for the existence of lipid rafts, the detection of these small and dynamic structures has been elusive. In 2009, Eggeling *et al.* used STED-FCS to provide convincing evidence for cholesterol-driven compartmentalization.[53] The subdiffraction lateral resolution of the STED beam creates a smaller focal volume for FCS analysis than a traditional confocal beam. This enhanced resolution made it possible to determine that sphingolipids and GPI-APs are transiently trapped in cholesterol-dependent nanodomains (<20 nm). Another technique that can improve resolution of the FCS volume is NSOM. NSOM provides improved axial as well as lateral resolution, creating an even smaller FCS volume than STED. With this approach, Manzo *et al.* have also detected heterogeneous behavior of sphingolipid diffusion that is consistent with compartmentalization.[54]

Detection of raft-marker association on the cell surface has also demonstrated the ability of lipids to organize membrane proteins. Using homo-FRET, several groups have detected small clusters of the raft-marker GPI-AP.[55,56] Bramshueber *et al.*[57] have examined GFP-GPI-AP and GM1 (lipid that marks raft domains) organization on living cells using an adaptation of FRAP to image the domains. They observed nanoscale clustering of these raft markers into stable platforms that were mobile and cholesterol-dependent. NSOM is capable of directly mapping out the nanoscale organization of the membrane with ~100 nm resolution[8] (see Figure 2). Using NSOM, Van Zanten *et al.*[20] found that cholera toxin-β (CTxB) binding to GM1 induces coalescence of CTxB-GM1 into nanodomains smaller than 120 nm. As expected, the classical nonraft marker CD71 did not colocalize with CTxB-GM1 and was randomly distributed. Interestingly, while raftophilic proteins (CD55, LFA-1, and GPI-AP) were found within close proximity to CTxB-GM1, the proteins did not mix, suggesting a recruitment of purported raft-associated proteins to GM1 that is stabilized by cholesterol-based interconnectivity. The clustering of proteins measured in these studies was found to be cholesterol-dependent, confirming cholesterol's key role in domain formation at the nanoscale.

Protein Islands. Douglas and Vale first demonstrated diffusional trapping of membrane proteins in discrete protein-defined microdomains.[58] Using two-color imaging, they tracked individual LAT-GFP or Lck-GFP motion with respect to total CD2-mRFP. LAT and Lck were seen to diffuse rapidly in the non-CD2 regions but undergo restricted diffusion upon entering a CD2-defined area, indicating that membrane proteins can be transiently trapped in membrane domains. In support of this evidence, electron micrographs of plasma membrane sheets labeled for signaling molecules revealed that proteins exist in distinct clusters surrounded by protein-free membrane.[3,59−61] Lillemeier and colleagues [3] termed these protein-rich regions "protein islands" and found that while all protein islands were enriched in cholesterol, some islands labeled with raft markers and others with non-raft markers. Additionally, the protein islands require actin for stability. Using high speed PALM (photoactivatable localization microscopy), TCR (T cell receptor) and LAT were shown to exist in stable yet distinct clusters on resting T cells, confirming in live cells that membrane proteins localize to distinct microdomains.[62] These observations indicate that membrane partitioning is more complex than defining raft/non-raft compartments.

Actin-Mediated Receptor Confinement. Single particle tracking studies have implicated membrane proximal actin structures in the formation of nanometer-sized "confinement zones" that restrict lateral diffusion.[4] Typically, these studies relied on chemical disruption of the actin cytoskeleton to correlate confinement with actin. In 2008, using two-color TIRF microscopy, the motion of individual quantum dot (QD)-tagged FcεRI was simultaneously imaged within the

Table 1. Fluorescence microscopy techniques for mapping of membrane organization or detecting protein−protein interactions

	brief description	quantitativeinformation	advantages	limitations
FRAP[70,100] [Fluorescence Recovery After Photobleaching]	Fluorophores are bleached in a small region and recovery of signal is monitored over time.	• Measures protein diffusion and immobile fraction	• Standard on any confocal microscope	• Low spatial and temporal resolution • Average protein diffusion
FRET[81,101,102] [Förster Resonance Energy Transfer]	Energy transfer between donor and acceptor labels is dependent on distance between them, typically 1−10 nm. ET measured by changes in donor intensity or lifetime.	• Detects interactions between two labeled proteins or conformational changes within a dual-labeled protein	• Single molecule FRET can be achieved at high speeds (<1 s) to measure binding events	• Fluorescence lifetime imaging (FLIM) is inherently slow • Donor/acceptor stoichiometry is critical
homoFRET[55,56,103]	Energy transfer between like fluorophores (*i.e.*, GFP to GFP), detected by changes in polarization anisotropy.	• Reports protein aggregation state	• Single class of fluorophore needed	• Overexpression of GFP-tagged proteins is required
SPT[63,65,67,100] [Single Particle Tracking]	Direct tracking of individual protein trajectories.	• Determines diffusion coefficients for each protein tracked • Reveals different modes of motion (free, restricted, immobile)	• High spatial (<50 nm) and temporal (>30 Hz) resolution • Multicolor SPT allows for distinguishing between multiple protein species	• Sparse labeling density required • Larger photostable probes (beads, QDs) have complications of multivalency • Small organic dyes are quickly photobleached
FCS[70,104] [Fluorescence Correlation Spectroscopy]	Analysis of intensity fluctuations generated as fluorescently tagged proteins diffuse in and out of a stationary focal volume.	• Protein diffusion coefficients • Protein concentration • Two-color cross-correlation detects protein complexes	• Measure diffusion of cytoplasmic and/or membrane proteins	• A single measurement takes seconds to minutes to complete • Will not detect immobile proteins • Sensitive to membrane fluctuations
ICS[105,106] [Image Correlation Spectroscopy]	Family of techniques similar to FCS, but intensity fluctuations are analyzed across an image or throughout a time series.	• Protein number density and aggregation state • Two-color cross-correlation detects protein complexes • Generates a map of protein behavior across the cell	• Can be measured with standard confocal or TIRF microscope • Faster temporal resolution can be reached using the time differences inherent in a confocal image due to raster scanning	• Measures average protein behavior, subpopulations are not distinguished
STED[53,93,107] [Stimulated Emission Depletion]	Gaussian excitation beam is overlaid with a doughnut-shaped beam that depletes emission from the outside ring of the excitation spot.	• Generates super-resolution images (30−100 nm)	• Two-color imaging is possible • Laser scanning technique with frame rates on the order of seconds	• Requires expensive, specialized equipment • Depletion beam uses high intensity
NSOM[8] [Near-field Scanning Optical Microscopy]	A scanned optical fiber excites and collects fluorescence near fiber tip.	• Mapping of protein or lipid distributions (~70 nm)	• Scanning tip also provides topography map • Multicolor imaging • Small focal volume eliminates cytosolic background	• Ultimate resolution requires fixed samples • Technically challenging technique • Probe fragility
Single Fluorophore Localization Microscopy[68,107]	Localization of individual molecules with high precision to build a high resolution image. Variants include PALM, STORM, and GSDIM.	• Generates super-resolution images (10−50 nm)	• sptPALM, live-cell PALM and STORM allow for imaging in live cells. • Multicolor imaging	• Ultimate resolution requires fixed samples • Data acquisition time can be long (min to hr) • Blinking/switching of organic dyes requires deoxygenated buffers

dx.doi.org/10.1021/cb200326g|ACS Chem. Biol. 2012, 7, 139−149

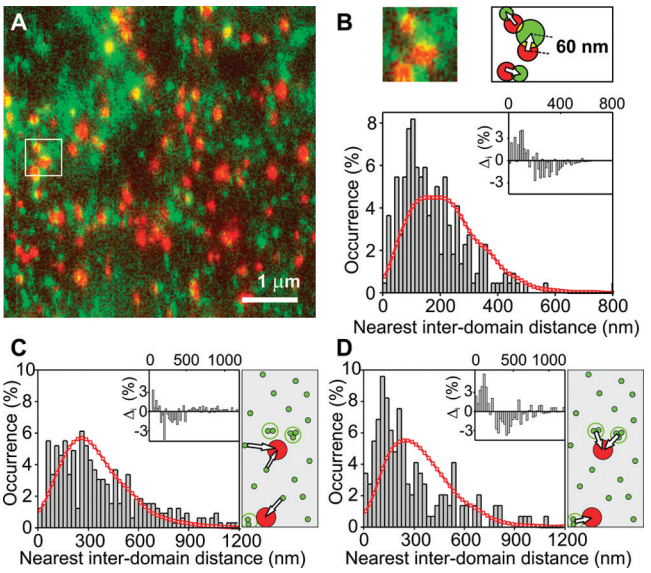


Figure 2. Mapping the membrane with NSOM. (A) Representative dual color excitation/detection NSOM image of LFA-1 integrin nanoclusters (red) and GPI-APs (green) at the cell surface of fixed monocytes in the absence of ligand. (B) The cartoon shows how the distance between the center of mass of a fluorescent spot and its nearest neighboring spot is calculated. Nearest interdomain distance distributions of LFA-1 nanoclusters to its closest GPI-AP (bars) together with simulations of random spatial distribution of LFA-1 nanoclusters with respect to GPI-APs (red). The inset corresponds to the difference (i in %) between experimental data and simulations. At shorter distances (crossover point in i) both distributions are significantly different with $P = 0.01$. These results demonstrated that LFA-1 nanoclusters, known to co-cap with large raft domains, are in fact spatially segregated but proximal to GPI-AP hotspots. Reproduced with permission from van Zanten *et al.*[108]

landscape of the membrane proximal GFP-actin bundles.[63] This directly showed for the first time that actin indeed acts as a physical barrier to transmembrane protein diffusion. These observations defined a larger-scale actin organization than those described by Kusumi and colleagues,[64] revealing a dynamic actin labyrinth with spatiotemporal scales on order of micrometers and seconds.

The ultimate question is whether this actin-dependent partitioning is a passive event or functions to alter protein behavior. Batista and colleagues have implicated actin as well as actin-binding proteins, ERM (ezrin-radixi-moesin), in regulation of B cell receptor (BCR) signaling. Using two-color TIRF microscopy, Treanor *et al.*[65] observed that resting BCR diffusion is restricted by both actin and ezrin structures. Interestingly, they also observed that BCR can be constrained within actin-rich regions, generating a population of receptors with reduced mobility. The disruption of the actin cytoskeleton induced cellular calcium response that correlated with increasing BCR diffusion. This work suggests a link between actin and ERM networks that partition receptors and prevent signaling. The partitioning may serve to sequester BCR from interactions partners such as active kinases or coreceptors. Alternatively, the compartmentalization may co-confine BCR with phosphotases that keep the receptor inactive. More recently, the same group has shown that ERM proteins are dephosphorylated upon BCR activation, which would alter BCR mobility and facilitate signaling.[66] Together, these results indicate an active interplay between BCR and the actin network that controls BCR signaling.

Actin compartmentalization may also influence signaling events by increasing protein interactions. Two-color single QD tracking revealed that actin co-confines receptors, promoting receptor encounters. In the FcεRI system, actin co-confines resting receptors, maintaining them in close proximity for extended periods of time, thereby increasing the local receptor concentration.[63] Actin was also shown to modulate FcεRI response to multivalent ligand binding, since disruption of actin increased the time for receptor immobilization upon antigen-induced cross-linking.[63] A corresponding cytoskeleton confinement has been observed for the immunoreceptor CD36 on macrophages. Jaqaman *et al.* showed that CD36 diffusion is constrained in linear channels that are actin- and microtubule-dependent.[67] Receptor co-confinement in these domains leads to an increased local density of receptors by ~5-fold and promotes transient interactions between unliganded receptors.

MEASURING PROTEIN DYNAMICS AND ORGANIZATION

Advancements in fluorescence microscopy techniques (Table 1) have made it possible to measure protein dynamics, aggregation state, and interactions on the living cell, facilitating measurements of biochemical parameters *in situ*. The family of image correlation techniques can determine average protein mobility, aggregation state, and protein−protein interactions based on ensemble measurements. Imaging techniques that circumvent the diffraction limit, including NSOM (Figure 2) and localization microscopy, are capable of directly mapping out the nanoscale organization of the membrane.[8,68] Single molecule imaging, such as FRET imaging and multicolor single particle tracking (Figure 3), provides a view of protein behavior at the molecular level.[69]

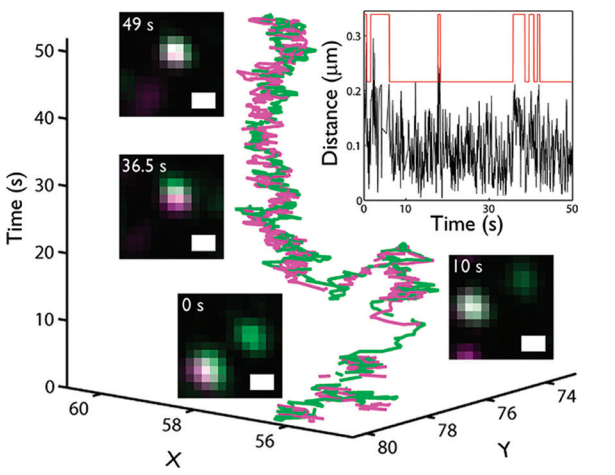


Figure 3. Capturing EGFR dimerization events. Tracking of QD585-EGF-EGFR (green) and QD655-EGF-EGFR (magenta) complexes. Trajectory over time shows close proximity of the two ligand-bound receptors with correlated motion. Insets: Stills from the acquired time series show moments of high colocalization and times when the receptors separate. Top right: Plot of distance between the two receptors as a function of time demonstrates fluctuations in separation. This is captured by the 3-state HMM that identifies repeated transitions (orange line) between dimer (D) and domain confined (C) states. Image courtesy of Shalini Low-Nam and similar to Low-Nam *et al.*[80]

dx.doi.org/10.1021/cb200326g | *ACS Chem. Biol.* 2012, 7, 139–149

Ensemble Measurements of Protein Organization. Classical microscopy techniques continue to provide insight into biological systems. For example, a recent paper by Itano *et al.* used two-color colocalization to show that the HIV-1 receptor DC-SIGN is organized in microdomains on the cell surface that are heterogeneous in composition, often mixing with other C-type lectins or clathrin.[70] These results support the previous findings of DC-SIGN localization to microdomains using electron microscopy or NSOM.[71,72] Itano *et al.* also combined FRAP, line scanning FCS, and single QD tracking to show that DC-SIGN does not exchange readily between microdomains and that the mobility within the domains is low. Together, these studies demonstrate the power of integrating data from disparate techniques that cross multiple time and length scales to create a more complete picture of a protein behavior.

Recently, the family of ICS techniques has grown rapidly with new approaches and analysis methods. In 2009, the Gratton group demonstrated the ability to detect protein complex stoichiometry and dynamics of exchange using cross-correlation Number & Brightness and RICS analysis.[73,74] Nagy *et al.*[75] used N&B to examine the distribution of EGFR on the plasma membrane. They found that EGFR exists as a monomer at low expression levels but forms ligand-independent dimers at higher levels of expression. Addition of EGF led to almost complete dimerization and the ultimate formation of higher order oligomers that associated with clathrin structures.

Spatial intensity distribution analysis (SpIDA) has recently been developed by the Wiseman group. This technique analyzes fluorescence images based on fitting intensity histograms to determine protein concentration and aggregation.[76] Since this analysis does not depend on spatial correlation, it can extract data from a single image and is not tied to the assumption that the sample is homogeneous. Swift *et al.*[77] applied SpIDA to simultaneously monitor EGFR dimerization and internalization (concentration at the membrane) in response to transactivation *via* GPCR. They found a differential response depending on the specific GPCR involved: all GPCR transactivation induced EGFR dimerization, yet not all of the GPCRs induced rapid EGFR internalization.

Capturing Single Molecule Behavior. The ensemble measurements described above considered the average, steady state characteristics of proteins but do not address the stochastic nature of receptor encounters, which can only be appreciated though single molecule observations. Recently, several groups have reported methods to monitor receptor interactions at the single molecule level and determine dimerization kinetics. Using the coincidence of Cy3B- and Alexa488-labeled ligands, Hern *et al.*[78] observed dimer formation and dissociation events as well as repeated interactions between the GPCR, M1 muscarinic acetylcholine receptor. Their results indicated that M1 exists in a dynamic equilibrium between monomer and dimer states. Analysis of the distribution of dimer durations determined an off rate of 1.3 s^{-1}. Kasai *et al.*[79] have used single molecule imaging to examine dimerization of another GPCR, the Formyl Peptide Receptor (FPR). In this work, they developed analysis methods to determine association and dissociation constants from single color data. They found that FPR also exists in a dynamic equilibrium with unliganded dimer lifetimes of 91 ms and found no significant change in the presence of ligand.

The previous two studies used organic dyes for labeling the GPCR. Low-Nam *et al.*[80] have used two-color QD tracking to characterize EGFR dimerization (Figure 3). The use of QDs allows for tracking of receptors over longer times without potential artifacts due to photobleaching. In this study, EGFR was either labeled through ligand (QD-EGF) or with a nonactivating camelid anti-erbB1 antibody fragment (QD-VHH). Since proteins are often co-confined in diffraction limited domains, correlated motion analysis was used to distinguish dimerization from colocalization. It was seen that resting receptors (QD-VHH) did not display correlated motion despite colocalization, while ligand-bound (QD-EGF) receptors demonstrated strong correlated motion. To quantify the kinetics of dimerization, a 3-state Hidden Markov Model was developed to extract transition rates between free, co-confined, and dimer states. The introduction of the co-confined state was required to accurately represent the data, indicating that co-confinement by microdomains plays an important role in receptor behavior. It was found that 2 ligand-bound receptors form more stable dimers than resting receptors, linking ligand occupancy to dimer stability. Furthermore, actin-based confinement was found to promote receptor dimerization.[80]

Single molecule techniques have also been developed to examine receptor–ligand interactions. Huppa *et al.*[81] used single molecule FRET (smFRET) to determine dissociation rates for the TCR-peptide-MHC complex in the context of the immunological synapse. When T cells with Cy3-scFv-labeled TCR (donor) were added to a planar lipid bilayer containing Cy5-peptide bound to MHC (pMHC), TCR-pMHC interactions resulted in measurable energy transfer between the FRET pair. The duration of the smFRET signal per interaction was quantified, and from this TCR-pMHC dissociation rate (K_d) was determined. Importantly, the K_d measured in intact cells during synapse formation was found to be much higher than those determined from *in vitro* measurements of purified protein, highlighting the importance of cellular geometry in modulating protein behavior.

OUTLOOK

More than a decade after the "discovery" of lipid rafts,[7,82] membrane compartmentalization is now a well-recognized general mechanism of regulating receptor function and signal transduction, and its many facets are becoming increasingly known. Clearly, cells exploit a variety of biochemical actions to modulate the aggregation of proteins and lipids at the nano- and microscale. Newly emerging post-translational modifications of membrane proteins are likely to play a yet unknown role in membrane organization. The interplay among these modifications most likely represents an additional level of signal transduction regulation.

Understanding of the coupling mechanism(s) between outer and inner domains is still in its infancy. It remains to be established how the outer and inner lipid layers are organized with respect to each other, and whether they concertedly contribute to the regulation of membrane-associated receptor signaling. Trans-membrane proteins as well as cholesterol could be involved in linking the biochemical information at both sides of the plasma membrane. Recent developments in the area of artificial lipid membrane preparation have shown that stable asymmetric giant unilamellar vesicles can be obtained that will be instrumental for unraveling the molecular mechanisms behind interleaflet coupling.[83]

At present, increasing evidence suggests that signaling nanodomains are also present in endosomes, putting forward the concept that endosomes are in fact intracellular signal

dx.doi.org/10.1021/cb200326g | *ACS Chem. Biol.* 2012, 7, 139–149

transduction stations.[84,85] Interestingly, Albi and co-workers have recently demonstrated the existence of lipid domains in the cell nucleus and their role in regulating enzymes involved in vitamin D3 uptake, therefore influencing cell differentiation.[86,87] Although the nanodomains present in nuclear membranes and endosomes are likely to play important roles in numerous cellular processes,[88] further investigation is needed to fully understand their properties and clarify their functions.

The current challenge in the membrane domain field is to understand how a cell integrates multiple biochemical strategies to induce, maintain, or modify membrane compartments to regulate signal transduction in time and space. While the fluorescence microscopy techniques described here provide information on protein behavior that would not be accessible through conventional biochemical techniques, even higher spatiotemporal resolution will be needed to address this challenge. Enhancements in spatial resolution have rapidly progressed in the past decade with advancements in super-resolution techniques such as STED,[68] PALM,[68] and GSDIM.[89] New advances in NSOM tip geometry are capable of enhancing the local electromagnetic fields to give ~30 nm axial resolution of protein localization on cell membranes.[90] Super-resolution techniques are starting to close the temporal gap with high frame rate techniques using PALM,[91,92] STED,[93] and structured illumination microscopy.[94] To understand the interplay between multiple proteins and lipids, higher multiplex imaging is needed. Hyperspectral microscopes that acquire the full emission spectrum of the sample rather than depending on filters[95] make it possible to increase the number of fluorophores that can be used to simultaneously image proteins and membrane markers. Many of these improvements depend not only on improved instrumentation but also on the generation of new fluorescent proteins, organic dyes, and fluorescent nanoparticles that will increase multicolor capability and allow for longer/faster live cell imaging. Lipid labeling remains a specific challenge, but new strategies are being developed that are bringing new options for imaging of nanodomains and lipid−protein interactions.[96−99] As imaging technologies continue to improve, cell biologists will be able to answer questions at spatiotemporal scales that were previously inaccessible. Integration of information from multiple disciplines, such as high resolution microscopy coupled to readouts of biochemical events, will ultimately provide a more complete description of how cell signaling is regulated.

AUTHOR INFORMATION

Corresponding Author

*E-mail: a.cambi@ncmls.ru.nl; dlidke@salud.unm.edu.

ACKNOWLEDGMENTS

We apologize to colleagues whose work could not be cited due to space limitations. We thank B. Wilson for critical reading of the manuscript and helpful suggestions and the reviewers for excellent suggestions. Work in our laboratories is supported by a Young Investigator Grant from the Human Frontier Science Program (HFSP) to D.S.L. and A.C.; A.C. is the recipient of a Meervoud grant (836.09.002) from The Netherlands Organisation for Scientific Research (NWO), and D.S.L. is supported by NSF CAREER Award MCB-0845062 and the UNM Spatiotemporal Modeling Center NIH P50GM085273.

KEYWORDS

Plasma membrane: The outer cell membrane made of a phospholipid bilayer that separates the cellular contents from the extracellular environment. Proteins embedded in the plasma membrane regulate responses to extracellular signals; Signal transduction: Activation of a membrane protein, by ligand binding or other cues, initiates a cascade of protein−protein interactions that propagates the signal to the nucleus and produces a physiological response; Protein biochemical modification: The chemical modification of a protein during or after its translation. This is achieved through the addition of biochemical functional groups such as lipids, carbohydrates, acetates, phosphates, *etc.*, that change the chemical nature of certain amino acids and extend the range of functions of the protein; Membrane partitioning: The formation of localized compartments on the plasma membrane due to lipid−lipid, lipid−protein, or protein−protein interactions; Lipid nanodomains: Favored interactions between certain types of lipids lead to their co-segregation in domains at the cell membrane; Actin Corrals: The restriction of membrane protein or lipid mobility by membrane proximal actin structure; Protein Islands: Diffusional trapping of membrane proteins in discrete protein-rich microdomains; Fluorescence microscopy: Optical imaging with submicrometer resolution using fluorescent-markers that allows for live cell imaging of dynamic cellular processes

REFERENCES

(1) Singer, S. J., and Nicolson, G. L. (1972) The fluid mosaic model of the structure of cell membranes. *Science 175*, 720−731.

(2) Pike, L. J. (2006) Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J. Lipid Res. 47*, 1597−1598.

(3) Lillemeier, B. F., Pfeiffer, J. R., Surviladze, Z., Wilson, B. S., and Davis, M. M. (2006) Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc. Natl. Acad. Sci. U.S.A. 103*, 18992−18997.

(4) Kusumi, A., Nakada, C., Ritchie, K., Murase, K., Suzuki, K., Murakoshi, H., Kasai, R. S., Kondo, J., and Fujiwara, T. (2005) 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. 34*, 351−378.

(5) van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol. 9*, 112−124.

(6) Lingwood, D., and Simons, K. (2010) Lipid rafts as a membrane-organizing principle. *Science 327*, 46−50.

(7) Simons, K., and Gerl, M. J. (2010) Revitalizing membrane rafts: new tools and insights. *Nat. Rev. Mol. Cell Biol. 11*, 688−699.

(8) van Zanten, T. S., Cambi, A., and Garcia-Parajo, M. F. (2010) A nanometer scale optical view on the compartmentalization of cell membranes. *Biochim. Biophys. Acta 1798*, 777−787.

(9) Baumgart, T., Hammond, A. T., Sengupta, P., Hess, S. T., Holowka, D. A., Baird, B. A., and Webb, W. W. (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3165−3170.

(10) Kiyokawa, E., Baba, T., Otsuka, N., Makino, A., Ohno, S., and Kobayashi, T. (2005) Spatial and functional heterogeneity of sphingolipid-rich membrane domains. *J. Biol. Chem. 280*, 24072−24084.

(11) Chen, Y., Qin, J., and Chen, Z. W. (2008) Fluorescence-topographic NSOM directly visualizes peak-valley polarities of GM1/GM3 rafts in cell membrane fluctuations. *J. Lipid Res. 49*, 2268−2275.

(12) Ira., and Johnston, L. J. (2008) Sphingomyelinase generation of ceramide promotes clustering of nanoscale domains in supported bilayer membranes. *Biochim. Biophys. Acta 1778*, 185−197.
(13) Zhang, Y., Li, X., Becker, K. A., and Gulbins, E. (2009) Ceramide-enriched membrane domains--structure and function. *Biochim. Biophys. Acta 1788*, 178−183.
(14) Niemela, P. S., Ollila, S., Hyvonen, M. T., Karttunen, M., and Vattulainen, I. (2007) Assessing the nature of lipid raft membranes. *PLoS Comput. Biol. 3*, e34.
(15) Zheng, H., Liu, W., Anderson, L. Y., and Jiang, Q. X. (2011) Lipid-dependent gating of a voltage-gated potassium channel. *Nat Commun 2*, 250.
(16) Coskun, U., Grzybek, M., Drechsel, D., and Simons, K. (2011) Regulation of human EGF receptor by lipids. *Proc. Natl. Acad. Sci. U.S.A. 108*, 9044−9048.
(17) Pontier, S. M., Percherancier, Y., Galandrin, S., Breit, A., Gales, C., and Bouvier, M. (2008) Cholesterol-dependent separation of the beta2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. *J. Biol. Chem. 283*, 24659−24672.
(18) Lingwood, D., Binnington, B., Rog, T., Vattulainen, I., Grzybek, M., Coskun, U., Lingwood, C. A., and Simons, K. (2011) Cholesterol modulates glycolipid conformation and receptor activity. *Nat. Chem. Biol. 7*, 260−262.
(19) Carroll-Portillo, A., Spendier, K., Pfeiffer, J., Griffiths, G., Li, H., Lidke, K. A., Oliver, J. M., Lidke, D. S., Thomas, J. L., Wilson, B. S., and Timlin, J. A. (2010) Formation of a mast cell synapse: Fc epsilon RI membrane dynamics upon binding mobile or immobilized ligands on surfaces. *J. Immunol. 184*, 1328−1338.
(20) van Zanten, T. S., Gomez, J., Manzo, C., Cambi, A., Buceta, J., Reigada, R., and Garcia-Parajo, M. F. (2010) Direct mapping of nanoscale compositional connectivity on intact cell membranes. *Proc. Natl. Acad. Sci. U.S.A. 107*, 15437−15442.
(21) Levental, I., Lingwood, D., Grzybek, M., Coskun, U., and Simons, K. (2011) Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proc. Natl. Acad. Sci. U.S.A. 107*, 22050−22054.
(22) Quinn, P. J. (2010) A lipid matrix model of membrane raft structure. *Prog. Lipid Res. 49*, 390−406.
(23) Di Paolo, G., and De Camilli, P. (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature 443*, 651−657.
(24) de Keijzer, S., Meddens, M. B., Kilic, D., Joosten, B., Reinieren-Beeren, I., Lidke, D. S., and Cambi, A. (2011) Interleukin-4 alters early phagosome phenotype by modulating Class I PI3K dependent lipid remodeling and protein recruitment. *PLoS One 6*, e22328.
(25) Yeung, T., Terebiznik, M., Yu, L., Silvius, J., Abidi, W. M., Philips, M., Levine, T., Kapus, A., and Grinstein, S. (2006) Receptor activation alters inner surface potential during phagocytosis. *Science 313*, 347−351.
(26) Lasserre, R., Guo, X. J., Conchonaud, F., Hamon, Y., Hawchar, O., Bernard, A. M., Soudja, S. M., Lenne, P. F., Rigneault, H., Olive, D., Bismuth, G., Nunes, J. A., Payrastre, B., Marguet, D., and He, H. T. (2008) Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. *Nat. Chem. Biol. 4*, 538−547.
(27) Levental, I., Grzybek, M., and Simons, K. (2010) Greasing their way: lipid modifications determine protein association with membrane rafts. *Biochemistry 49*, 6305−6316.
(28) Varma, R., and Mayor, S. (1998) GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature 394*, 798−801.
(29) Levental, I., Lingwood, D., Grzybek, M., Coskun, U., and Simons, K. (2010) Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proc. Natl. Acad. Sci. U.S.A. 107*, 22050−22054.
(30) Resh, M. D. (2006) Trafficking and signaling by fatty-acylated and prenylated proteins. *Nat. Chem. Biol. 2*, 584−590.
(31) Farazi, T. A., Waksman, G., and Gordon, J. I. (2001) The biology and enzymology of protein N-myristoylation. *J. Biol. Chem. 276*, 39501−39504.
(32) Wan, J., Roth, A. F., Bailey, A. O., and Davis, N. G. (2007) Palmitoylated proteins: purification and identification. *Nat. Protoc. 2*, 1573−1584.
(33) Henis, Y. I., Hancock, J. F., and Prior, I. A. (2009) Ras acylation, compartmentalization and signaling nanoclusters (Review). *Mol. Membr. Biol. 26*, 80−92.
(34) Bijlmakers, M. J. (2009) Protein acylation and localization in T cell signaling (Review). *Mol. Membr. Biol. 26*, 93−103.
(35) Porter, J. A., Young, K. E., and Beachy, P. A. (1996) Cholesterol modification of hedgehog signaling proteins in animal development. *Science 274*, 255−259.
(36) Schey, K. L., Gutierrez, D. B., Wang, Z., Wei, J., and Grey, A. C. (2010) Novel fatty acid acylation of lens integral membrane protein aquaporin-0. *Biochemistry 49*, 9858−9865.
(37) Foster, M. W., Hess, D. T., and Stamler, J. S. (2009) Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol. Med. 15*, 391−404.
(38) Ibiza, S., Perez-Rodriguez, A., Ortega, A., Martinez-Ruiz, A., Barreiro, O., Garcia-Dominguez, C. A., Victor, V. M., Esplugues, J. V., Rojas, J. M., Sanchez-Madrid, F., and Serrador, J. M. (2008) Endothelial nitric oxide synthase regulates N-Ras activation on the Golgi complex of antigen-stimulated T cells. *Proc. Natl. Acad. Sci. U.S.A. 105*, 10507−10512.
(39) Raines, K. W., Cao, G. L., Lee, E. K., Rosen, G. M., and Shapiro, P. (2006) Neuronal nitric oxide synthase-induced S-nitrosylation of H-Ras inhibits calcium ionophore-mediated extracellular-signal-regulated kinase activity. *Biochem. J. 397*, 329−336.
(40) Thibeault, S., Rautureau, Y., Oubaha, M., Faubert, D., Wilkes, B. C., Delisle, C., and Gratton, J. P. (2010) S-nitrosylation of beta-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol. Cell 39*, 468−476.
(41) Into, T., Inomata, M., Nakashima, M., Shibata, K., Hacker, H., and Matsushita, K. (2008) Regulation of MyD88-dependent signaling events by S nitrosylation retards toll-like receptor signal transduction and initiation of acute-phase immune responses. *Mol. Cell. Biol. 28*, 1338−1347.
(42) Baker, T. L., Booden, M. A., and Buss, J. E. (2000) S-Nitrosocysteine increases palmitate turnover on Ha-Ras in NIH 3T3 cells. *J. Biol. Chem. 275*, 22037−22047.
(43) Raju, K., Doulias, P. T., Tenopoulou, M., Greene, J. L., and Ischiropoulos, H. (2011) Strategies and tools to explore protein S-nitrosylation, *Biochim. Biophys. Acta* E-pub ahead of print; DOI: 10.1016/j.bbagen.2011.05.009.
(44) Spange, S., Wagner, T., Heinzel, T., and Kramer, O. H. (2009) Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int. J. Biochem. Cell Biol. 41*, 185−198.
(45) Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science 325*, 834−840.
(46) Ma, L., Gao, J. S., Guan, Y., Shi, X., Zhang, H., Ayrapetov, M. K., Zhang, Z., Xu, L., Hyun, Y. M., Kim, M., Zhuang, S., and Chin, Y. E. (2010) Acetylation modulates prolactin receptor dimerization. *Proc. Natl. Acad. Sci. U.S.A. 107*, 19314−19319.
(47) Rabinovich, G. A., Toscano, M. A., Jackson, S. S., and Vasta, G. R. (2007) Functions of cell surface galectin-glycoprotein lattices. *Curr. Opin. Struct. Biol. 17*, 513−520.
(48) Lajoie, P., Goetz, J. G., Dennis, J. W., and Nabi, I. R. (2009) Lattices, rafts, and scaffolds: domain regulation of receptor signaling at the plasma membrane. *J. Cell Biol. 185*, 381−385.
(49) Demotte, N., Stroobant, V., Courtoy, P. J., Van Der Smissen, P., Colau, D., Luescher, I. F., Hivroz, C., Nicaise, J., Squifflet, J. L., Mourad, M., Godelaine, D., Boon, T., and van der Bruggen, P. (2008) Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity 28*, 414−424.
(50) Nieminen, J., Kuno, A., Hirabayashi, J., and Sato, S. (2007) Visualization of galectin-3 oligomerization on the surface of

neutrophils and endothelial cells using fluorescence resonance energy transfer. *J. Biol. Chem. 282*, 1374−1383.
(51) Demetriou, M., Granovsky, M., Quaggin, S., and Dennis, J. W. (2001) Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature 409*, 733−739.
(52) Lajoie, P., Partridge, E. A., Guay, G., Goetz, J. G., Pawling, J., Lagana, A., Joshi, B., Dennis, J. W., and Nabi, I. R. (2007) Plasma membrane domain organization regulates EGFR signaling in tumor cells. *J. Cell Biol. 179*, 341−356.
(53) Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., Belov, V. N., Hein, B., von Middendorff, C., Schonle, A., and Hell, S. W. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature 457*, 1159−1162.
(54) Manzo, C., van Zanten, T. S., and Garcia-Parajo, M. F. (2011) Nanoscale fluorescence correlation spectroscopy on intact living cell membranes with NSOM probes. *Biophys. J. 100*, L8−10.
(55) Bader, A. N., Hofman, E. G., Henegouwen, P. M. P. V. E., and Gerritsen, H. C. (2007) Imaging of protein cluster sizes by means of confocal time-gated fluorescence anisotropy microscopy. *Optics Express 15*, 6934−6945.
(56) Sharma, P., Varma, R., Sarasij, R. C., Ira., Gousset, K., Krishnamoorthy, G., Rao, M., and Mayor, S. (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell 116*, 577−589.
(57) Brameshuber, M., Weghuber, J., Ruprecht, V., Gombos, I., Horvath, I., Vigh, L., Eckerstorfer, P., Kiss, E., Stockinger, H., and Schutz, G. J. (2010) Imaging of mobile long-lived nanoplatforms in the live cell plasma membrane. *J. Biol. Chem. 285*, 41765−41771.
(58) Douglass, A. D., and Vale, R. D. (2005) Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell 121*, 937−950.
(59) Wilson, B. S., Pfeiffer, J. R., and Oliver, J. M. (2002) FcepsilonRI signaling observed from the inside of the mast cell membrane. *Mol. Immunol. 38*, 1259−1268.
(60) Prior, I. A., Muncke, C., Parton, R. G., and Hancock, J. F. (2003) Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J. Cell Biol. 160*, 165−170.
(61) Yang, S., Raymond-Stintz, M. A., Ying, W., Zhang, J., Lidke, D. S., Steinberg, S. L., Williams, L., Oliver, J. M., and Wilson, B. S. (2007) Mapping ErbB receptors on breast cancer cell membranes during signal transduction. *J. Cell Sci. 120*, 2763−2773.
(62) Lillemeier, B. F., Mortelmaier, M. A., Forstner, M. B., Huppa, J. B., Groves, J. T., and Davis, M. M. (2010) TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat. Immunol. 11*, 90−96.
(63) Andrews, N. L., Lidke, K. A., Pfeiffer, J. R., Burns, A. R., Wilson, B. S., Oliver, J. M., and Lidke, D. S. (2008) Actin restricts FcepsilonRI diffusion and facilitates antigen-induced receptor immobilization. *Nat. Cell Biol. 10*, 955−963.
(64) Kusumi, A., Sako, Y., and Yamamoto, M. (1993) Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys. J. 65*, 2021−2040.
(65) Treanor, B., Depoil, D., Gonzalez-Granja, A., Barral, P., Weber, M., Dushek, O., Bruckbauer, A., and Batista, F. D. (2010) The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity 32*, 187−199.
(66) Treanor, B., Depoil, D., Bruckbauer, A., and Batista, F. D. (2011) Dynamic cortical actin remodeling by ERM proteins controls BCR microcluster organization and integrity. *J. Exp. Med. 208*, 1055−1068.
(67) Jaqaman, K., Kuwata, H., Touret, N., Collins, R., Trimble, W. S., Danuser, G., and Grinstein, S. (2011) Cytoskeletal control of CD36 diffusion promotes its receptor and signaling function. *Cell 146*, 593−606.
(68) Toomre, D., and Bewersdorf, J. (2010) A new wave of cellular imaging. *Annu. Rev. Cell Dev. Biol. 26*, 285−314.
(69) Lidke, D. S., and Wilson, B. S. (2009) Caught in the act: quantifying protein behaviour in living cells. *Trends Cell Biol. 19*, 566−574.
(70) Itano, M. S., Neumann, A. K., Liu, P., Zhang, F., Gratton, E., Parak, W. J., Thompson, N. L., and Jacobson, K. (2011) DC-SIGN and influenza hemagglutinin dynamics in plasma membrane microdomains are markedly different. *Biophys. J. 100*, 2662−2670.
(71) de Bakker, B. I., de Lange, F., Cambi, A., Korterik, J. P., van Dijk, E. M., van Hulst, N. F., Figdor, C. G., and Garcia-Parajo, M. F. (2007) Nanoscale organization of the pathogen receptor DC-SIGN mapped by single-molecule high-resolution fluorescence microscopy. *ChempPhysChem 8*, 1473−1480.
(72) Cambi, A., de Lange, F., van Maarseveen, N. M., Nijhuis, M., Joosten, B., van Dijk, E. M., de Bakker, B. I., Fransen, J. A., Bovee-Geurts, P. H., van Leeuwen, F. N., Van Hulst, N. F., and Figdor, C. G. (2004) Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. *J. Cell Biol. 164*, 145−155.
(73) Digman, M. A., Wiseman, P. W., Choi, C., Horwitz, A. R., and Gratton, E. (2009) Stoichiometry of molecular complexes at adhesions in living cells. *Proc. Natl. Acad Sci. U.S.A. 106*, 2170−2175.
(74) Digman, M. A., Wiseman, P. W., Horwitz, A. R., and Gratton, E. (2009) Detecting protein complexes in living cells from laser scanning confocal image sequences by the cross correlation raster image spectroscopy method. *Biophys. J. 96*, 707−716.
(75) Nagy, P., Claus, J., Jovin, T. M., and Arndt-Jovin, D. J. (2010) Distribution of resting and ligand-bound ErbB1 and ErbB2 receptor tyrosine kinases in living cells using number and brightness analysis. *Proc. Natl. Acad. Sci. U.S.A. 107*, 16524−16529.
(76) Godin, A. G., Costantino, S., Lorenzo, L. E., Swift, J. L., Sergeev, M., Ribeiro-da-Silva, A., De Koninck, Y., and Wiseman, P. W. (2011) Revealing protein oligomerization and densities *in situ* using spatial intensity distribution analysis. *Proc. Natl. Acad. Sci. U.S.A. 108*, 7010−7015.
(77) Swift, J. L., Godin, A. G., Dore, K., Freland, L., Bouchard, N., Nimmo, C., Sergeev, M., De Koninck, Y., Wiseman, P. W., and Beaulieu, J. M. (2011) Quantification of receptor tyrosine kinase transactivation through direct dimerization and surface density measurements in single cells. *Proc. Natl. Acad. Sci. U.S.A. 108*, 7016−7021.
(78) Hern, J. A., Baig, A. H., Mashanov, G. I., Birdsall, B., Corrie, J. E., Lazareno, S., Molloy, J. E., and Birdsall, N. J. (2010) Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc. Natl. Acad. Sci. U.S.A. 107*, 2693−2698.
(79) Kasai, R. S., Suzuki, K. G., Prossnitz, E. R., Koyama-Honda, I., Nakada, C., Fujiwara, T. K., and Kusumi, A. (2011) Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J. Cell Biol. 192*, 463−480.
(80) Low-Nam, S. T., Lidke, K. A., Cutler, P. J., Roovers, R. C., vanvBergen en Henegouwen, P. M. P., Wilson, B. S., and Lidke, D. S. (2011) ErbB1 dimerization is promoted by domain co-confinement and stabilized by ligand-binding, *Nat. Struct. Mol. Biol.* Epub ahead of print; DOI: 10.1038/nsmb.2135.
(81) Huppa, J. B., Axmann, M., Mortelmaier, M. A., Lillemeier, B. F., Newell, E. W., Brameshuber, M., Klein, L. O., Schutz, G. J., and Davis, M. M. (2010) TCR-peptide-MHC interactions *in situ* show accelerated kinetics and increased affinity. *Nature 463*, 963−967.
(82) Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature 387*, 569−572.
(83) Chiantia, S., Schwille, P., Klymchenko, A. S., and London, E. (2011) Asymmetric GUVs prepared by MbetaCD-mediated lipid exchange: an FCS study. *Biophys. J. 100*, L1−3.
(84) Fehrenbacher, N., Bar-Sagi, D., and Philips, M. (2009) Ras/MAPK signaling from endomembranes. *Mol. Oncol. 3*, 297−307.
(85) Schenck, A., Goto-Silva, L., Collinet, C., Rhinn, M., Giner, A., Habermann, B., Brand, M., and Zerial, M. (2008) The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. *Cell 133*, 486−497.

(86) Bartoccini, E., Marini, F., Damaskopoulou, E., Lazzarini, R., Cataldi, S., Cascianelli, G., Gil Garcia, M., and Albi, E. (2011) Nuclear lipid microdomains regulate nuclear vitamin D3 uptake and influence embryonic hippocampal cell differentiation. *Mol. Biol. Cell 22*, 3022−3031.
(87) Cascianelli, G., Villani, M., Tosti, M., Marini, F., Bartoccini, E., Magni, M. V., and Albi, E. (2008) Lipid microdomains in cell nucleus. *Mol. Biol. Cell 19*, 5289−5295.
(88) Scita, G., and Di Fiore, P. P. (2010) The endocytic matrix. *Nature 463*, 464−473.
(89) Lalkens, B., Testa, I., Willig, K. I., Hell, S. W. (2011) MRT letter: Nanoscopy of protein colocalization in living cells by STED and GSDIM, *Microsc. Res. Tech.* Epub ahead of print; DOI: 10.1002/jemt.21026.
(90) van Zanten, T. S., Lopez-Bosque, M. J., and Garcia-Parajo, M. F. (2010) Imaging individual proteins and nanodomains on intact cell membranes with a probe-based optical antenna. *Small 6*, 270−275.
(91) Shroff, H., Galbraith, C. G., Galbraith, J. A., and Betzig, E. (2008) Live-cell photoactivated localization microscopy of nanoscale adhesion dynamics. *Nat. Methods 5*, 417−423.
(92) Manley, S., Gillette, J. M., Patterson, G. H., Shroff, H., Hess, H. F., Betzig, E., and Lippincott-Schwartz, J. (2008) High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat. Methods 5*, 155−157.
(93) Hein, B., Willig, K. I., and Hell, S. W. (2008) Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell. *Proc. Natl. Acad. Sci. U.S.A. 105*, 14271−14276.
(94) Kner, P., Chhun, B. B., Griffis, E. R., Winoto, L., and Gustafsson, M. G. (2009) Super-resolution video microscopy of live cells by structured illumination. *Nat. Methods 6*, 339−342.
(95) Sinclair, M. B., Haaland, D. M., Timlin, J. A., and Jones, H. D. (2006) Hyperspectral confocal microscope. *Appl. Opt. 45*, 6283−6291.
(96) Haberkant, P., Schmitt, O., Contreras, F. X., Thiele, C., Hanada, K., Sprong, H., Reinhard, C., Wieland, F. T., and Brugger, B. (2008) Protein-sphingolipid interactions within cellular membranes. *J. Lipid Res. 49*, 251−262.
(97) Haberkant, P., and van Meer, G. (2009) Protein-lipid interactions: paparazzi hunting for snap-shots. *Biol. Chem. 390*, 795−803.
(98) Hannoush, R. N., and Arenas-Ramirez, N. (2009) Imaging the lipidome: omega-alkynyl fatty acids for detection and cellular visualization of lipid-modified proteins. *ACS Chem. Biol. 4*, 581−587.
(99) Yap, M. C., Kostiuk, M. A., Martin, D. D., Perinpanayagam, M. A., Hak, P. G., Siddam, A., Majjigapu, J. R., Rajaiah, G., Keller, B. O., Prescher, J. A., Wu, P., Bertozzi, C. R., Falck, J. R., and Berthiaume, L. G. (2010) Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry. *J. Lipid Res. 51*, 1566−1580.
(100) Marguet, D., Lenne, P. F., Rigneault, H., and He, H. T. (2006) Dynamics in the plasma membrane: how to combine fluidity and order. *EMBO J. 25*, 3446−3457.
(101) Jares-Erijman, E. A., and Jovin, T. M. (2003) FRET imaging. *Nat. Biotechnol. 21*, 1387−1395.
(102) Robia, S. L., Campbell, K. S., Kelly, E. M., Hou, Z., Winters, D. L., and Thomas, D. D. (2007) Forster transfer recovery reveals that phospholamban exchanges slowly from pentamers but rapidly from the SERCA regulatory complex. *Circ. Res. 101*, 1123−1129.
(103) Tramier, M., and Coppey-Moisan, M. (2008) Fluorescence anisotropy imaging microscopy for homo-FRET in living cells. *Methods Cell Biol. 85*, 395−414.
(104) He, H. T., and Marguet, D. (2011) Detecting nanodomains in living cell membrane by fluorescence correlation spectroscopy. *Annu. Rev. Phys. Chem. 62*, 417−436.
(105) Digman, M. A., and Gratton, E. (2011) Lessons in fluctuation correlation spectroscopy. *Annu. Rev. Phys. Chem. 62*, 645−668.
(106) Kolin, D. L., and Wiseman, P. W. (2007) Advances in image correlation spectroscopy: Measuring number densities, aggregation states, and dynamics of fluorescently labeled macromolecules in cells. *Cell Biochem. Biophys. 49*, 141−164.
(107) Leung, B. O., and Chou, K. C. (2011) Review of super-resolution fluorescence microscopy for biology. *Appl. Spectrosc. 65*, 967−980.
(108) van Zanten, T. S., Cambi, A., Koopman, M., Joosten, B., Figdor, C. G., and Garcia-Parajo, M. F. (2009) Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. *Proc. Natl. Acad. Sci. U.S.A. 106*, 18557−18562.