Uncovering homo-and hetero-interactions on the cell membrane using single particle tracking approaches

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Abstract
The plasma membrane of eukaryotic cells is responsible for a myriad of functions that regulate cell physiology and plays a crucial role in a multitude of processes that include adhesion, migration, signaling recognition and cell–cell communication. This is accomplished by specific interactions between different membrane components such as lipids and proteins on the lipid bilayer but also through interactions with the underlying cortical actin cytoskeleton on the intracellular side and the glycocalyx matrix in close proximity to the extracellular side. Advanced biophysical techniques, including single particle tracking (SPT) have revealed that the lateral diffusion of molecular components on the plasma membrane represents a landmark manifestation of such interactions. Indeed, by studying changes in the diffusivity of individual membrane molecules, including sub-diffusion, confined diffusion and/or transient arrest of molecules in membrane compartments, it has been possible to gain insight on the nature of molecular interactions and to infer on its functional role for cell response. In this review, we will revise some exciting results where SPT has been crucial to reveal homo- and hetero-interactions on the cell membrane.

Keywords: single particle tracking, anomalous diffusion, cell membrane compartmentalization, actin–cytoskeleton interactions, clathrin interactions, glycan-based interactions

(Some figures may appear in colour only in the online journal)
Indeed, the current view of the cell membrane is that of a highly heterogeneous structure with multiple compartments spanning a broad range of spatial and temporal scales. Moreover, molecules within these compartments often exhibit deviation of their mobility from a purely Brownian behavior, such as anomalous diffusion [5, 7–11].

The quantification of molecular diffusivity has fundamental importance in studying the function of membrane components. This is because mobility is often affected by interactions between the molecule under study and its surroundings. Thus, by studying the lateral diffusion of a protein (or lipid) it is possible to gain insight into the occurrence of molecular interactions and to infer on its functional role for cell response. A broad range of experimental techniques have been developed and applied during the past decades for measuring the lateral mobility of molecules on the cell membrane. One of the most widespread techniques used by biologists for nearly four decades is fluorescence recovery after photobleaching (FRAP), reviewed in [12, 13]. FRAP is based on the photobleaching of a given area on the cell membrane with intense laser illumination. By monitoring the recovery of the fluorescence within the photobleached area in time, the overall mobility of the fluorescently labeled molecules can be retrieved with a moderate temporal resolution. Fluorescence correlation spectroscopy (FCS) is another technique, which similar to FRAP, allows characterizing the diffusion of fluorescently labeled molecules, reviewed in [14, 15]. In FCS, the fluorescence emission of molecules diffusing through a fixed illumination volume (usually confocal) is recorded over a given period of time. By analyzing the fluctuation of the fluorescence intensity over time, the number of molecules and the diffusion coefficient, among other parameters, can be extracted. Although FRAP and FCS have the sufficient temporal resolution (in the case of FCS even below milliseconds) to monitor fast dynamic processes, their spatial resolution is limited by diffraction when using standard confocal microscopy. As such, FRAP and FCS provide ensemble time average of the dynamics of the molecules under study. Therefore, nanoscale heterogeneities within the cell membrane that affect the diffusion of the molecules, and/or short-lived molecular interaction events are in general not accessible with these methods.

An alternative approach to FRAP and FCS, which features high temporal resolution and single molecule sensitivity is single particle tracking (SPT), reviewed in [9, 16–18]. The technique is based on the ability of detecting the motion of individual molecules over time with millisecond time resolution. The large majority of SPT experiments are commonly performed by localizing the position of individual proteins labeled with fluorescent markers, rendering spatial localization precisions in the order of tens of nanometers. Nevertheless, it is worth mentioning that non-fluorescent approaches based on light scattering of large particles can also be used for SPT. More recently, interferometric scattering microscopy (iSCAT) has been implemented as an alternative highly sensitive technique able to localize the position of individual label-free proteins with nanometer precision [19, 20]. By reconnecting the coordinates of each individual localization as a function of time, trajectories can be generated and, by their analysis, the diffusion of individually labeled proteins or lipids can be characterized [21–24]. In the last two decades SPT has been extensively used to reveal and to characterize homo- and hetero-interactions on the cell membrane. In this review, we will summarize salient findings from the literature where SPT has been instrumental to reveal such molecular interactions and to infer on their role for cellular function. Importantly, these interactions are often reflected as changes in the diffusivity of the molecule under study that alter its motion from a purely Brownian behavior. SPT has provided a great amount of detail in terms of different types of motion experienced by proteins and lipids as they diffuse on the plasma membrane. In the last part of this review we will summarize recent efforts towards the application and development of new theoretical models aimed at describing anomalous diffusion on the plasma membrane of living cells.

2. Dynamic homo-interactions on the cell membrane

A first example of interactions affecting molecular diffusion consists of homophilic interactions, in which membrane molecules transiently or stably interact with identical molecules leading to the formation of supramolecular structures such as dimers or oligomers. These types of interactions are rather relevant for a large family of signaling proteins, including the epidermal growth factor receptor (EGFR) and the G-protein coupled receptor (GPCR). The occurrence of receptor dimerization or oligomerization prior to ligand binding or upon external stimulation (e.g. ligand binding) and its role on intracellular signaling have been widely debated and addressed by means of SPT.

EGFRs are one of the four types of membrane tyrosine kinases present in the human body [25, 26]. The EGFR family is involved in regulating signaling activities in a wide range of cellular processes such as cell growth and differentiation [25, 27]. Increased expression of EGFR has been found in a large number of cancers including breast, brain or lung cancer [28, 29]. Earlier crystallography data suggested that ligand-induced dimerization of the EGFR was necessary to trigger its signaling activity [26, 30–32]. SPT of Cy3/Cy5 labeled EGF in combination with single molecule fluorescence resonance energy transfer (FRET) allowed the successful monitoring for the first time of the dynamic formation of EGFR dimers on the cell membrane upon EGF binding [33]. Interestingly, this study also showed the existence of pre-formed EFGR dimers even before ligand binding. The dynamic formation of EGFR dimers was also observed in a later study using single color quantum dot (QD) tracking [34]. By analyzing diffusivity changes, the authors also observed that EGFR spontaneously formed short-lived dimers before ligand addition. Although the kinetic stability of unliganded dimers was in principle sufficient for EGF-independent activation, ligand binding was found to be required for signaling [34]. These findings were further confirmed in another study by using dual-color QD tracking of EGFR molecules labeled with an equimolar solution of EGF-QD655 and -QDS85 conjugates [35]. Dual-color
SPT allowed direct visualization of the correlated motion of two differently labeled EGFRs when transiently forming diffusing dimers on the cell membrane (figures 1(a),(b)). This elegant work unequivocally showed the highly transient nature of pre-formed dimers, i.e. in the absence of the ligand, and that dimer stability is directly linked to ligand occupancy. Interestingly, these studies also showed a strong dependence of the EGFR dimerization rate on the actin cytoskeleton (figures 1(c),(d)), suggesting that cytoskeletal confinement could favor the encounter of EGFRs to enhance their dimerization [35].

The GPCR family provides another notable example of membrane receptor homo-interactions in the context of cell signaling. Genetic analysis has determined that GPCRs constitute the largest family of membrane proteins in mammals [36, 37] and they are responsible for a large amount of signal transduction in the human body, including several fundamental functions such as vision, neurotransmission or hormone-induced physiological reactions [38–40]. Therefore, GPCRs have been one of the main targets of a large number of drugs produced by the pharmaceutical industry [36]. Similar to EGFR, there has been a long debate as to whether GPCRs are present on the cell membrane as monomers, dimers or higher-order oligomers [41]. The first direct evidence of the homodimerization state of a GPCR at the single molecule level was achieved by combining total internal reflection fluorescence microscopy (TIRFM) with single-color and dual-color SPT on living cells [42]. The authors showed that the M1 muscarinic acetylcholine receptor transiently forms dimers with an inter-conversion from monomer to dimer within seconds. Moreover, the percentage of dimers was estimated to be ~30% [42]. In another study, SPT and quantitative fluorescence microscopy were applied to determine the dimer association/dissociation rate of a GPCR [43]. The conclusion of this study also pointed towards a continuous and very dynamic inter-conversion between monomers and dimers of GPCRs, with monomers converting into dimers every 150 ms and dimers dissociating into monomers in ~90 ms [43].

The use of SPT has also brought new insights for cell signaling in the immune system. In this context, it is becoming clear that oligomerization of membrane receptors constitutes a fundamental mechanism to regulate the initial stages of the immune response at the molecular level [4]. The occurrence of homo-interactions aimed to promote or stabilize signaling platforms has been described for a number of membrane receptors responsible for the identification of pathogenic components such as the T cell receptor [44–46], the B cell receptor [47, 48] (BCR) or the FceRI receptor [49, 50]. Several roles have been proposed for these receptor oligomers, such as an increased ability for binding to multimeric ligands [4, 51] or serving as signaling scaffolds on the cell membrane [4, 52, 53]. Some examples of immune-receptor oligomerization are described below.

Multi-color QD tracking has been applied to obtain mechanistic insight on homo-interactions of FceRI receptors [49, 50]. The authors showed that ligand-dependent interactions between FceRI receptors were promoted by their co-confinement within the actin cytoskeleton meshwork. In the resting state, actin confinement was insufficient to induce FceRI oligomerization as no correlated motion between FceRI receptors was observed. However, after ligand addition, FceRI formed aggregates whose size and mobility depended on the
concentration and multivalency of the ligand. Importantly, the authors showed that the actin cytoskeleton enhanced the immobilization kinetics of cross-linked FcεRI receptors, indicating that the actin cytoskeleton promotes the encountering rate of FcεRI thereby resulting in ligand-induced aggregation [49, 50]. Interestingly, dual color SPT experiments have suggested a similar operating mechanism for the class B scavenger receptor 36 (CD36) on the surface of macrophages [54]. The experiments showed that CD36 displays movement along linear trajectories as a result of its confinement within channels determined by underlying microtubules. Such confinement promotes the encountering rate of CD36 and leads to an enhanced CD36 metastable clustering in the basal state. The authors suggested that such preclustering could lead to a higher response in the case of a multivalent mediated ligand activation of CD36 compared to monomeric CD36 [54].

The oligomerization state of the B-Cell receptor (BCR) has been also investigated by combining SPT with super-resolution fluorescence techniques [48]. In the basal state, the BCR was found to organize in nanoclusters on the plasma membrane of B cells, whose mobility is tightly regulated by the actin cytoskeleton [48]. More recently, it was reported that Toll-like receptor (TLR) stimulation compromises the integrity of the actin cytoskeleton resulting in increased mobility of the BCR on the cell membrane. This led to a higher collision rate of BCRs, which in turn induced a higher signaling activity as compared to un-stimulated cells. The authors concluded that TLR stimulation sensitizes B cell activation by promoting BCR homo-interactions on the plasma membrane of B cells [55].

The combination of SPT and super-resolution microscopy has also demonstrated the inherent oligomerization state of the pathogen recognition receptor DC-SIGN and its relevance for virus pathogen capture and internalization [56]. Previous studies using electron-microscopy showed that DC-SIGN formed nanoclusters on the surface of resting immature dendritic cells (imDCs) [57]. Moreover, DC-SIGN nanoclustering resulted crucial for HIV-capture and binding by imDCs [57]. Recently, we revealed that truncation of the extracellular neck region, known to abrogate DC-SIGN tetramerization [58], significantly reduced nanoclustering and concomitantly increased lateral diffusion. Importantly, DC-SIGN nanocluster dissolution exclusively compromised binding to nanoscale size pathogens [56]. These results thus underscore a direct relationship between spatial nanopatterning, driven by intracellular interactions between the neck regions, and receptor diffusion to provide DC-SIGN with the exquisite ability to dock pathogens at the virus length scale.

It is important to mention that membrane tension is gaining considerable attention in recent years as an additional mechanism capable of regulating protein homo- and hetero-interactions on the cell membrane. For instance, SPT of two transmembrane proteins reconstituted in artificial membranes showed that the mobility of membrane proteins crucially depend on the local membrane deformation self-generated around the protein, which can be tuned by adjusting membrane tension [59]. This local deformation induced by membrane tension can then drive the formation of homo-interactions for those proteins that are sensitive to curvature. Disruption of membrane tension can also lead to the re-arrangement of spatial organization of receptors on the cell membrane and the formation of heteromeric complexes as recently demonstrated in the case of TGFβ receptors in living cells [60].

3. Dynamic hetero-interactions on the cell membrane

Over the last 20 years, a large number of biophysical studies performed by means of different techniques—including SPT—has evidenced that interactions between different molecular components, i.e. hetero-interactions, take place at the cell membrane strongly influence the diffusion, organization and eventually the function of many receptor proteins. Although these studies have identified the existence of several binding molecular partners, two main structures, namely the actin cytoskeleton and lipid rafts, have been found to directly or indirectly affect receptor diffusion through heterophilic interactions. This has led to postulate these structures as fundamental membrane organizing principles as reviewed in [5]. Although membrane proteins can in principle interact with both kinds of structures, in this section we review the two concepts somewhat separately, describing their role in membrane compartmentalization and how this affects cellular function.

3.1. Hetero-interactions with the actin cytoskeleton

The actin cytoskeleton is based on microfilaments of fibrous actin (F-Actin) which are composed of polymerized monomeric globular actin (G-Actin) [61]. The notion that the actin cytoskeleton can interact with different proteins located at the cell membrane was already proposed more than three decades ago by Sheetz and co-workers [62]. Using SPT, Kusumi and colleagues showed in 1993 that the diffusion of proteins and lipids on the cell membrane is transiently hindered by the presence of the underlying actin meshwork forming compartments with a characteristic size of 200–500 nm [10]. Although molecular diffusion appears to be hindered by the presence of these actin compartments, molecules can in principle also ‘hop’ between adjacent compartments (hop-diffusion model). To support this hypothesis, in a later study, it was shown that the lateral movement of the transferrin and α2-macroglobulin receptor was transiently confined within compartments for ≈29 s. Importantly, the mobility within the compartments could be described as purely Brownian diffusion [63, 64]. Disruption of the actin cytoskeleton increased the amount of trajectories classified as Brownian diffusion leading to the proposal that the actin cytoskeleton is responsible of such dynamic confinement [64]. Using different cytoplasmic tail mutants, the same authors showed that the actin cytoskeleton could also regulate the lateral mobility of E-Cadherin by sterically hindering its cytoplasmic tail while diffusing on the cell membrane [65]. Besides membrane proteins, the actin cytoskeleton has been proposed to also indirectly interact with membrane lipids affecting their lateral diffusion. In this context, Kusumi and co-workers showed cytoskeleton-dependent
hop-diffusion of the outer leaflet phospholipid DOPE on the plasma membrane of NRK fibroblasts [66]. Since outer leaflet lipids cannot physically interact with the cortical actin cytoskeleton, the authors proposed a model (the so-called picket-fence model), where the motion of molecules on the cell membrane can be hindered by cytoskeleton anchored-proteins spanning the membrane leaflets [66].

Aside from these indirect interactions, the cortical actin cytoskeleton has been also implicated in direct interactions regulating the diffusion and function of different membrane receptors. One clear example is provided by integrin receptors involved in cell adhesion. Using SPT, several groups, including ours, have shown that the actin cytoskeleton regulates the lateral mobility of the integrin receptor $\alpha_\beta_2$ (also known as LFA-1) [67, 68]. Moreover, it was found that LFA-1 conformational state is strongly coupled to its lateral mobility, with the open and ligated forms of the integrin strongly interacting with actin, promoting its immobilization on the cell membrane [67, 68]. These studies suggested that the actin cytoskeleton controls the adhesiveness of LFA-1 expressing cells by coupling the conformational state of the integrin to its lateral mobility. In a somewhat related work, Rossier et al investigated the dynamic nanoscale organization of $\beta_1$ and $\beta_3$ integrins [69]. Using SPT and super-resolution imaging the authors showed that these integrins reside in focal adhesions (FA) through free-diffusion and immobilization cycles. Integrin activation promoted its immobilization, which was stabilized in FAs by simultaneous connection to the ligand and actin-binding proteins, indicating once more that activation of integrins requires anchoring to the actin cytoskeleton.

A major breakthrough in studying the role of the actin cytoskeleton was achieved by dual-color imaging, allowing the recording of SPT trajectories with respect to fluorescently labeled actin. As already described in the previous section, Lidke and colleagues visualized for the first time the lateral mobility of individual receptors (QD labeled FcRRI) together with GFP-labeled actin [49]. SPT has been also applied to directly visualize the interaction of the BCR with the actin cytoskeleton being tagged with lifeact-GFP [48] (figure 2(a)). By tracking the BCR inside and outside actin rich regions, the authors showed that BCR mobility inside actin rich regions was reduced as compared to other regions, providing clear evidence of a physical interaction of the BCR with the actin cytoskeleton (figures 2(b),(c)). Moreover, deletion of the BCR cytoplasmic tail increased its mobility, suggesting that the coupling of the BCR to the actin cytoskeleton is a key regulator of B cell tonic signaling [48].

3.2. Hetero-interactions with lipid rafts

While it is clear that the actin cytoskeleton can regulate the diffusion of membrane proteins either by indirect and direct interactions, it is legitimate to ask whether similar effects can be also induced by the main components of the cell membrane, i.e. the lipids themselves. In particular, a strongly debated membrane organizing structure consists in the so-called lipid rafts. Lipid rafts are thought to be small and dynamic nanodomains composed of self-associated sphingolipids and cholesterol, constantly nucleating and dissociating on the cell membrane and able to oligomerize ‘raftophilic’ proteins such as, for example, glycosylphosphatidylinositol-anchored proteins (GPI-APs) [70–74]. However, due to their small size and highly dynamic nature, lipid rafts have been so far very difficult to visualize in living cells. SPT has played a major role to indirectly study such structures, via e.g. measuring changes in diffusion behavior of putative raft interactions. Using an algorithm capable of detecting transient confinement zones of diffusing molecules [75, 76], it was reported that GPI-APs were transiently confined on the cell membrane [77]. Since the degree of confinement was reduced in sphingolipid depleted cells or in M\(\beta\)CD treated cells, the authors concluded that the source for such transient confinements could be the partitioning of GPI-APs in cholesterol-dependent lipid rafts [77, 78]. A more direct visualization of the influence of lipid rafts into the lateral diffusion of GPI-APs was further obtained using single color SPT together with dual-color fluorescence microscopy [79]. This work showed that the lateral mobility of GPI-APs was reduced when GPI-APs were found in the proximity of cholesterol-rich GM1 domains.

It is important to mention that other factors, aside from lipid raft partitioning, have been also invoked to explain the transient confinement and oligomerization state of GPI-APs, as different homo- and hetero-interactions have been reported in the literature. For instance, by combining SPT with single molecule sensitive FRET, Kusumi’s group recently showed that ectodomain interactions are the primary factor in transiently stabilizing homodimers of the GPI-AP CD59, with cholesterol and other lipids having secondary stabilizing roles [80]. Ligation of CD59 promoted the formation of stable oligomer rafts containing up to four CD59 molecules. These oligomers depended on GPI anchorage and cholesterol and were capable of triggering intracellular Ca\(^{2+}\) responses.

GPI-APs have been also observed to interact with the actin cytoskeleton, although the exact mechanism is still under heavy debate. For instance, the picket-fence model proposed by Kusumi suggests that GPI-APs might be corralled in compartments created by the proximal actin cytoskeleton restricting their lateral diffusion and favoring their mutual interaction [81, 82]. A different model, proposed by the Mayor’s group suggests that cortical actin is directly responsible for the oligomerization of GPI-APs [83]. In this model short actin filaments would form aster-like aggregates under the plasma membrane that would actively drive GPI-APs into higher-order oligomers, with cholesterol playing only a marginal role [84]. This hypothesis has received a lot of attention from the community although, so far the existence of actin asters has been difficult to prove given their small size and presumably short lifetime.

SPT has been also crucial in determining the role of lipid rafts in tuning cell signaling, by crosslinking or clustering of GPI-APs. By artificially cross-linking GPI-APs, it was shown that lipid rafts were necessary to initiate a signaling event
involving SFKs, PI3 kinases and caveolin-1 [85]. Using a similar methodology, it was shown that cholesterol was involved in the transient immobilization of pre-crosslinked CD59 on the cell membrane [86, 87]. Moreover, Go12, Lyn and PLCγ2 were recruited within to the immobilized CD59 nanoclusters, which then produced cytosolic bursts of IP3-Ca2+2. Such mechanism for signal transduction led the authors to propose a ‘summation model’ where individual digital-like bursts of IP3 production, induced by single molecule events, might be sufficient to generate a bulk IP3 signal in the cytosol when integrated in time [86]. Notably, the single molecule sensitivity and high temporal resolution provided by SPT were fundamental to provide evidences that finally resulted in this novel signaling concept.

Figure 2. Direct visualization of interactions between the actin cytoskeleton and the BCR on B cells mapped with dual color SPT. (a) Selected dual-color TIRFM image to simultaneously visualize Lifeact-GFP (green) and track single molecules of the BCR isotype IgM (red) in B cells. The images in the right panels are magnified time sequences of the left panel (white square) with an example of a 2D trajectory of IgM indicated in yellow, with the diffusing particle outlined with a white circle. (b) Magnified image showing trajectories of IgM inside (red) and outside (yellow) actin-rich regions (gray-scale). The enlarged images on the middle and right panels show actin-rich regions, demarcated by white lines. Trajectories inside actin rich areas are shown in the middle panel and color-coded in red, while trajectories outside actin rich regions are showed in yellow in the right panel. (c) Scatterplot (left) and histogram (right) of the diffusion coefficients of IgM inside (circles) and outside (triangles) actin-rich areas with the median indicated by red bar. IgM motion is highly restricted in actin-rich regions indicating direct interaction between the BCR and the actin cytoskeleton. Scale bar represents 2 μm. Adapted from figure 3 of [48] with permission.
However, other studies using SPT have shown unrestricted diffusion of some GPI-APs on the plasma membrane of different cells, challenging the hypothesis that lipid rafts and/or the actin cytoskeleton could influence the lateral diffusion and interactions of lipid-anchored proteins. For instance, by using a minimally invasive labeling approach, the Schuetz group convincingly showed un-confined diffusion of the GPI-anchored protein CD59, indicating that its motion is not restricted by periodic cytoskeletal barriers [88], in contrast to earlier results obtained by Kusumi’s Lab [89]. More recently, the same group showed that CD59 does not interact with or partition on micropatterns of GPI-APs induced on living cell membranes [90]. By combining a protein micropatterning technique with SPT, the authors re-arranged GPI-APs directly in living cell plasma membranes and measured the recruitment and mobility of CD59 inside and outside the GPI-AP enriched regions. Interestingly, they found that CD59 did not show any preferential recruitment to the GPI-AP regions and that mobility was not influenced by the presence of cholesterol or temperature. Instead, the reduction of CD59 mobility could be fully explained by the presence of obstacles brought about by the increased local density of GPI-APs, questioning previous models of lipid rafts and/or actin barriers regulating the diffusion and interactions between GPI-APs.

3.3. Hetero-interactions with other membrane components

So far the application of SPT to the study of membrane proteins has mainly revealed changes in diffusion induced by interactions with lipid rafts and/or the actin cytoskeleton. Lately however, the increased use of SPT (in particular in its multicolor version) has enabled real-time visualization of receptor interactions with other membrane components such as clathrin-coated pits and the glycoalyx matrix, showing also the effects of these molecular components affecting the diffusion and regulating the function of membrane proteins.

Clathrin represents the main endocytic route of cargo proteins in mammalian cells, reviewed in [91]. Therefore, clathrin constitutes a major interaction partner of diffusing cargo proteins on the cell membrane. Recently, the interaction of the voltage-gated potassium channels Kv2.1 and Kv1.4 with clathrin-coated pits (CCPs) has been studied using dual-color SPT [92] (figure 3(a)). Interestingly, the authors showed that interactions between these cargo proteins and clathrin occurred transiently and followed a ‘catch-and-release’ mechanism (figure 3(b)). The distribution of the immobilization times of the receptor within CCPs displayed a long tail, which could be fitted with a power-law distribution function (figure 3(c)). Moreover, the probability of the cargo escaping from the CCP decreased as a function of the time spent in the pit. Since the probability of a new cargo entering into a previously occupied pit decreased with the number of cargo molecules contained within the pit, the authors suggested that the catch-and-release mechanism by CCPs could be a regulatory process of the cell to carefully select which cargo needs to be internalized (figure 3(d)). In addition, as the distribution of immobilization times displayed a power-law tail at long times, the authors proposed cargo-clathrin interactions as a potential source for anomalous diffusion on the cell membrane.

An additional membrane component that is starting to receive considerable attention from the biophysics community is the extracellular glycoalyx matrix. Glycans are fundamental cellular components ubiquitously present in the extracellular matrix and cell membrane as glycoproteins or glycolipids. Glycan-binding proteins such as galectins, siglecs, and selectins are mostly multivalent and thus thought to cross-link glycoproteins into higher-order aggregates, creating a cell surface glycan-based connectivity also called the glycan lattice or network, reviewed in [93, 94]. These galectin-glycan lattices have been shown to control a large number of cellular processes in the immune system such as pathogen-host interactions or even anti-tumor immunity responses [93, 95, 96]. Although their functional role has been studied for several years, the influence of glycan interactions on the mobility of the receptors has only started to be addressed by SPT and super-resolution microscopy approaches.

Using dual-color SPT and super-resolution STED, we recently visualized the impact of glycan-based interactions on the spatiotemporal organization and interaction with clathrin of the glycosylated pathogen recognition receptor DC-SIGN [97] (discussed in section 1) (figure 4). We found that cell surface glycan-mediated interactions did not influence the nanoscale lateral organization of DC-SIGN but restricted the mobility of the receptor to distinct micrometer-size membrane regions (figure 4(a)). Remarkably, these regions were also enriched in clathrin (figure 4(b)). The meso-scale compartmentalization of the receptor brought about by the glycan network increased the probability of DC-SIGN–clathrin interactions beyond random encountering (figures 4(c),(d)). Removal of the N-glycosylation site of DC-SIGN or neutralization of the glycan network by the use of lactose led to larger membrane exploration of the receptor and reduced interaction with clathrin, compromising clathrin-dependent internalization of virus-like particles by DC-SIGN. These results thus demonstrated that the glycosylation-based connectivity constitutes a new layer of membrane organization at the micron-scale complementary to those induced by the actin cytoskeleton and lipid rafts.

A similar kind of spatial regulation by the glycan network has been seen to influence integrin organization that ultimately modulated intracellular signaling in ways that can contribute to cancer metastasis on cancer cells [98]. Using a combination of biochemical and different biophysical approaches including SPT, the authors showed that the presence of long synthetic glycolectins or the natural glycoprotein mucin-1 resulted in an expanded membrane–extracellular matrix gap, clustering of integrins, exclusion of glycopolymers from regions of integrin adhesion, and membrane bending. These physical effects altered cell signaling through the MEK, PI3K and FAK pathways, leading to enhanced cell survival. This work thus further supports the notion that, in addition to their biochemical properties, the constituents of the glycoalyx can physically influence the spatial organization of cell-surface receptors and hence their activity. So far, spatial regulation by the glycoalyx
has been observed on DC-SIGN and integrins, but these effects are likely to be common to other cell-surface receptors that are regulated by receptor clustering or related intermolecular interactions. We expect that in the future, much research will focus on visualizing the glycocalyx matrix and identifying its role regulating the diffusion, organization and interaction of many membrane proteins. Along these lines, current efforts are focusing on the imaging of surface glycans using metabolic labeling and detection with CuAAC-based click chemistry approaches, a method developed by Bertozzi’s group [99, 100]. Using these dye-labeled glycans in mammary cancer cells, recent super-resolution and SPT studies revealed constrained diffusion of both N- and O-linked glycans [101, 102]. These results have been interpreted as reflecting the mobility of the glycans inside regions of pronounced galectins binding rather than to be caused by transient immobilization owing to spatial inhomogeneities on the plasma membrane [101].

4. Anomalous diffusion as a result of multi-molecular interactions

Many of the examples provided in the previous sections prove the importance of SPT in determining, either directly or indirectly, the effect of interactions occurring between the molecule under observation and the surrounding environment. These interactions can produce changes in diffusivity, transient trapping or local confinement that alter the particle motion from a pure Brownian behavior and are reflected in the observables of an SPT experiment. Generally speaking, the first signature of the occurrence of molecular interactions affecting the particle’s motion is observed in the dependence of the mean-square displacement (MSD) with respect to the time lag [18, 103–106]. In contrast to a pure Brownian diffusion—producing a linear dependence of the MSD over the time lag—membrane molecules often exhibit directed motion, confined motion,
anomalous diffusion and transient immobilization, as reviewed in [18, 104], giving rise to a nonlinear scaling of the MSD. As such, the quantitative study of the MSD can provide a first test to probe potential particle interactions with the environment.

In order to interpret these different behaviors and exploit single particle trajectories to obtain more detailed information on the particle interactions with the surrounding media, a considerable theoretical effort has been made by means of both numerical [107, 108] and analytical methods [109–114]. The most largely studied case is anomalous diffusion, i.e. a type of diffusion producing a power law scaling of the MSD $\sim t^{\alpha}$. The value of the anomalous exponent $\alpha$ allows further distinguishing between subdiffusion ($0 < \alpha < 1$) and superdiffusion ($\alpha > 1$). Anomalous diffusion has been widely observed both intracellularly and at cell membranes, and generally associated to molecular crowding [115] producing obstacles and/or traps to diffusion [18]. Although several theoretical models have been proposed for the interpretation of anomalous diffusion from SPT experiments [107, 108, 111, 116, 117], addressing the mechanism causing the anomalous behavior is often not straightforward and requires dedicated experiments and thorough analysis.

In recent years, the use of highly photo-stable fluorescent probes, in particular QDs, has opened the possibility of recording long single-particle trajectories (of the order of hundreds to thousand of time points) allowing better detection of anomalous diffusive behavior and thorough investigation of its causes [21, 49, 79, 92, 97]. Interestingly, some of these experiments have highlighted the occurrence of anomalous diffusion in connection to an exotic physical phenomenon, namely ergodicity breaking [118, 119].

According to the ergodic hypothesis, time and ensemble averages of a physical observable are equivalent. This means that an observable (e.g. the MSD) calculated on a single particle for a long time is equivalent to the same observable calculated over a large number of particles at a given time. However, even when tracking chemically identical molecules diffusing in cells, it has been found that the time-averaged MSD (tMSD), i.e. the MSD calculated for each different particle, shows a large scattering of values [118–123], reflecting differences either among the particles themselves, the space they explore, or the interactions they experience. Therefore, time averages remain random variables and hence irreproducible [118, 119, 121–123], leading to the breakdown of the central limit theorem [111]. As a consequence, the ensemble-averaged MSD (eMSD) displays a different scaling with respect to the tMSD and thus produces ergodicity breaking. The occurrence of ergodicity breaking implies that the molecules under investigation, although chemically identical, show somehow different dynamic properties. These dynamic properties can reflect a permanent state of the molecule, i.e. a situation in which the system’s phase space is constituted by non-connected domains and each particle trajectory explores only one of them, giving rise to the so-called ‘strong’ ergodicity breaking. However, nonergodicity can also manifest itself in a more elusive manner, referred to as ‘weak’ ergodicity breaking, as first introduced by Bouchaud for physical glasses [109]. In the ‘weak’ case, the molecules can change dynamic state and thus—in principle—explore the whole phase space.

However, if the whole space has an infinite exploration time, a single trajectory will never explore all the possible dynamic states, no matter how long one measures [124]. The occurrence of weak ergodicity breaking is characterized by the presence of aging, i.e. a power law dependence of an observable on

![Figure 4](image-url). Glycan-based interactions promote interactions between clathrin and the glycosylated pathogen recognition receptor DC-SIGN. (a) Single molecule localization events obtained from dual color SPT movies and collapsed in a reconstructed image that shows regions of the plasma membrane dynamically explored by DC-SIGN. The upper row shows wt-DC-SIGN (left) and de-glycosylated mutant (N80A) expressed on CHO cells. Lower row shows the dynamic exploration of DC-SIGN on untreated (left) and lactose-treated imDCs (right). Glycan-based interactions with DC-SIGN maintain the receptor confined in meso-scale regions of the cell membrane (~1 μm in size). Receptor de-glycosylation or removal of lectins by lactose abrogate glycan interactions and lead to a broader and un-restricted membrane exploration by the receptor. (b) Dual color images of clathrin super-imposed with dynamic localizations of wt-DC-SIGN-QD (left) and N80A-QD (right). White arrows indicate colocalization of DC-SIGN in CCP regions. (c) Frequency of localizations of DC-SIGN with respect to the clathrin signal. Corralling of wt-DC-SIGN in meso-scale compartments of the cell membrane brings the receptor closer to clathrin-rich regions, favoring their interaction. Adapted from figures 3 and 4 of [97] with permission.
the measurement time T. In the case of single particle trajectories, this observable is represented by the time-ensemble-averaged MSD (teMSD), obtained by sequentially averaging over both the time and the particles. Trajectories experiencing weak ergodicity breaking thus display a power law scaling of teMSD with respect to the measurement time T [111], with an exponent which does not depend on the time lag.

Weak ergodicity breaking has been observed experimentally for intracellular diffusion [123, 125] and two recent studies have evidenced its occurrence also on the cell membrane [118, 119]. The first example of weak ergodicity breaking on the cell membrane was provided by Weigl and coworkers, who studied the lateral diffusion of the voltage-gated potassium channel Kv2.1 on the membrane of human embryonic kidney cells [119]. In analyzing the diffusion of the Kv2.1 channel, the authors discovered that tMSD values at each time lag were scattered largely in contrast to eMSD values, which instead displayed a narrower distribution. This first evidence of nonergodicity was further supported by the observation of aging, revealed by a dependence of the teMSD on the measurement time T. These findings were interpreted by means of the continuous-time-random walk (CTRW) model, a generalization of the classical random walk [126] in which the waiting time and/or the step length might be continuous random variables. In this specific case, the motion of the Kv2.1 channel was compatible with that of a CTRW-like particle performing consecutive jumps with a power-law distribution of waiting times between jumps \( t^{-1(1+\beta)} \).

In a biophysical context, this type of CTRW can be interpreted as a motion in the presence of traps [108], e.g. due to biochemical binding of the diffuser with immobile structures. To test this hypothesis, the authors calculated the distribution of the time needed for the channel to escape from circles with different radii. This analysis confirmed that the escape times were power-law distributed and that this behavior was nearly independent on the circle radius, as expected for CTRW-like particle immobilization. Notably, when perturbing the actin cytoskeleton with Cytochalasin D or Swinholide A, the diffusion of the Kv2.1 recovered the ergodic behavior, strongly suggesting hetero-interactions between the potassium channel and the actin cytoskeleton as the main responsible for the nonergodic behavior. Although these results showed that the diffusion of the Kv2.1 channel was weakly nonergodic and compatible with the CTRW model, an observed feature could not be fully described by the CTRW alone. In fact, molecules performing a CTRW would still show a linear behavior in the tMSD, whereas the experiments showed a subdiffusive behavior tMSD \( \sim \tau^{\alpha} \) with \( \alpha \approx 0.8 \). The authors solved this discrepancy by assuming the coexistence of nonergodic dynamics, driven by binding to actin cytoskeleton, and an ergodic mechanism, attributable to molecular crowding and responsible for the observed anomalous diffusion.

Our group has recently provided a second example of nonergodic diffusion on the cell membrane by studying the diffusion of the pathogen recognition receptor DC-SIGN on the plasma membrane of stably transfected CHO cells [118]. The tMSDs of DC-SIGN were also largely scattered but scaled linearly with time lag. However, the eMSD and the teMSD showed a power law (eMSD \( \sim \tau^\beta \)) revealing that DC-SIGN diffusion also exhibits weak ergodicity breaking. However, in contrast to the observations on the KV2.1 channel, DC-SIGN experienced very few transient immobilization events during the total recording time, a feature that is incompatible with the CTRW model. The inadequacy of the CTRW model to describe the diffusion of DC-SIGN was further confirmed by the escape time analysis which, in contrast again to KV2.1 channel, displayed a marked dependence on the circle radius. Interestingly, further analysis of the trajectories by means of a change-point algorithm [127] revealed that a large fraction of DC-SIGN molecules experienced sudden changes in diffusivity. These results led to the hypothesis that the heterogeneous membrane environment could be sufficient to cause the weak ergodicity breaking of DC-SIGN diffusion through a variety of interactions with other intra- and extra-cellular membrane components that would continuously change the diffusivity of the receptor. To test this hypothesis, we developed a theoretical model in which a diffuser can randomly choose a diffusion coefficient from a probability distribution \( P(D) \) and experience this diffusivity value for a random time \( \tau \), after which a new diffusivity value is drawn. Making quite general assumptions on the form of \( P(D) \) (i.e. \( P(D) \sim D^{-\gamma-1} \) for \( D \rightarrow 0 \) and \( P(D) \) decaying fast to zero for \( D \rightarrow \infty \)) and on the relationship between \( D \) and \( \tau \) (i.e. \( E[\tau|D] \sim D^{-\gamma} \)) we have shown that weak nonergodic behavior indeed emerges for values of the exponents \( \gamma > \sigma \). Numerical simulations faithfully reproduced all the features of DC-SIGN nonergodic dynamics. Moreover, we also assessed the role of the structure of DC-SIGN on its diffusion by means of a mutational approach. Notably, we found that deletion of an extracellular structural motif (the so-called neck region) lead to a recovery of pure Brownian and ergodic diffusion. Since we previously showed that this mutation also impairs DC-SIGN homo-interactions and virus-binding capability [56], these experiments showed a direct correlation between DC-SIGN nonergodicity and its pathogen-recognition function. However, understanding whether correlation also implies causation, as well as understanding the molecular causes of nonergodicity requires further investigation.

These two examples of exotic diffusion suggest that spatiotemporal complexity, in the form of pathologically long immobilization times or space/time-dependent diffusivity, generates ergodicity breakdown. This phenomenon could represent a new form of response of complex systems to external stimuli. In fact, deviations from Brownian diffusion could not only reflect homo- and hetero-interactions on the cell membrane but could also translate to cellular functions, such as receptor endocytosis, cell adhesion or signaling.

5. Conclusions

The current picture of the plasma membrane is that of a highly heterogeneous structure with multiple compartments spanning a broad range of spatial and temporal scales. Molecules within the cell membrane interact with each other and with their immediate neighbors on the proximal intracellular and extracellular space resulting in changes on their lateral motion. We have summarized in here a few examples in...
which deviations from Brownian motion of molecules have provided evidence for homo- and hetero-interactions on the cell membrane. In a cell biology context, such interactions are crucial to control the function of many proteins and receptors, for example those involved in signaling, adhesion, pathogen recognition, immune response, etc. At the same time, the study of lateral diffusion in such a complex environment with multiple interactions between different molecular players has lead to the discovery of anomalous diffusion, sub-diffusion and ergodicity breaking on the cell membrane. Although the amount of research on the cell membrane continues at an accelerated pace, the combination of new techniques such as super-resolution microscopy in conjunction with advanced developments in SPT, will enable even more discoveries of molecular interactions in the next few years. This will certainly be accompanied by the development of new theoretical models to understand the complex behavior of cell membranes and the emergence of collective phenomena. As such, we predict that studies on the cell membrane will continue to be an exciting research field both for cell biologists as well as for biophysicists.

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References

[1] Edidin M 2010 Immunol. Rev. 276 65–72
[2] Frye L and Edidin M 1970 J. Cell. Sci. 7 319–35
[3] Singer S and Nicolson G L 1972 Science 175 720–31
[4] Garcia-Parajo M F, Cambi A, Torreno-Pina J A, Thompson N and Jacobson K 2014 J. Cell Sci. 127 4995–5005
[5] Kusumi A, Suzuki K G, Kasai R S, Ritchie K and Fujwara T K 2011 Trends Biochem. Sci. 36 604–15
[6] Nicolson G L 2014 Biochim. Biophys. Acta 1838 1451–66
[7] Edidin M, Zuniga M C and Sheetz M P 1994 Proc. Natl Acad. Sci. USA 91 3378–82
[8] Jacobson K, Sheets E D and Simson R 1995 Science 268 1441
[9] Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, Murakoshi H, Kasai R S, Kondo J and Fujwara T 2005 Annu. Rev. Biophys. Biomol. Struct. 34 351–78
[10] Kusumi A, Sako Y and Yamamoto M 1993 Biophys. J. 65 2021–40
[11] Sheetz M P, Schindler M and Koppel D E 1980 Nature 285 510–2
[12] Lippincott-Schwartz J, Altan-Bonnet N and Patterson G H 2003 Nat. Cell Biol. 5 57–14
[13] Reits E A and Neefjes J J 2001 Nat. Cell Biol. 3 E145–7
[14] Bacia K, Kim S A and Schwille P 2006 Nat. Methods 3 83–9
[15] Haustein E and Schwille P 2007 Annu. Rev. Biophys. Biomol. Struct. 36 151–69
[16] Kusumi A, Tsunooyama T A, Hiroswawa K M, Kasai R S and Fujwara T K 2014 Nat. Chem. Biol. 10 524–32
[17] Pinaud F, Clarke S, Sittner A and Dahan M 2010 Nat. Methods 7 725–85
[18] Saxton M J and Jacobson K 1997 Annu. Rev. Biophys. Biomol. Struct. 26 373–99
[19] Ortega-Arroyo J, Andrecka J, Spillane K M, Billington N, Takagi Y, Sellers J R and Kukura P 2014 Nano Lett. 14 2065–70
[20] Ortega-Arroyo J and Kukura P 2012 Phys. Chem. Chem. Phys. 14 15625–36
[21] Dahan M, Levi S, Luccardini C, Rostaing P, Riveau B and Triller A 2003 Science 302 442–5
[22] Jaqaman K 2008 Nat. Methods 5 695–702
[23] Schmidt T, Schütz G, Baumgartner W, Gruber H and Schindler H 1996 Proc. Natl. Acad. Sci. U S A 93 2926–9
[24] Sergé A, Bertaux N, Rigneault H and Marguet D 2008 Nat. Methods 5 687–94
[25] Lemmon M A and Schlessinger J 2010 Cell 141 1117–34
[26] Schlessinger J 2002 Cell 110 669–72
[27] Zhang X, Gureasko J, Shen K, Cole P A and Kuriyan J 2006 Cell 125 1137–49
[28] Sharma S V, Bell D W, Settleman J and Haber D A 2007 Nat. Rev. Cancer 7 169–81
[29] Zhang Z, Stiegler A L, Boggon T J, Kobayashi S and Halmos B 2010 Oncotarget 1 497
[30] Endres N F et al 2013 Cell 152 543–56
[31] Jura N, Endres N F, Engel K, Deindl S, Das R, Lamers M H, Wemmer D E, Zhang X and Kuriyan J 2009 Cell 137 1293–307
[32] Klein P, Mattoo D, Lemmon M A and Schlessinger J 2004 Proc. Natl Acad. Sci. USA 101 929–34
[33] Sako Y, Minoghehni S and Yanagida T 2004 Nat. Cell Biol. 2 168–72
[34] Chung I, Akita R, Vandlen R, Toomre D, Schlessinger J and Mellman I 2010 Nature 464 783–7
[35] Low-Nam S T, Lidke K A, Roovers R C, van Bergen en Henegouwen P M, Wilson B S and Lidke D S 2011 Nat. Struct. Mol. Biol. 18 1244–9
[36] Kohlbitz B K 2007 Biochim. Biophys. Acta 1768 794–807
[37] Ritter S L and Hall R A 2009 Nat. Rev. Mol. Cell Biol. 10 819–30
[38] Palczewski K 2006 Annu. Rev. Biochem. 75 743
[39] Venkatakrishnan A, Deupi X, Lebon G, Tate C G, Schertler G F and Babu M M 2013 Nature 494 185–94
[40] Watkins H A, Au M and Hay D L 2012 Drug Discov. Today 17 1006–14
[41] Kasai R S and Kusumi A 2014 Curr. Opin. Cell Biol. 27 78–86
[42] Hern J A, Baig A H, Mashanov G I, Birdsell B, Corrie J E, Lazareno S, Molloy J E and Birdsell N J 2010 Proc. Natl Acad. Sci. USA 107 2693–8
[43] Kasai R S, Suzuki K G, Prossnitz E R, Koyama-Honda I, Nakada C, Fujwara T K and Kusumi A 2011 J. Cell Biol. 192 463–80
[44] Lillemeier B F, Mörtelmaier M A, Forstner M B, Huppa J, Groves J T and Davis M M 2010 Nat. Immunol. 11 90–6
[45] Schamel W W and Alarcón B 2013 Immunol. Rev. 251 13–20
[46] Schamel W W, Arechaga I, Rüssel O R, van Santen H M, Caberas P, Risco C, Valpuesta J M and Alarcón B 2005 J. Exp. Med. 202 493–503
[47] Mattila P K et al 2013 Immunity 38 461–74
[48] Treanor B, Depoil D, Gonzalez-Granja A, Barral P, Weber M, Dushek O, Bruckbauer A and Batista F D 2010 Immunity 32 187–99
[49] Andrews N L, Lidke K A, Pfeiffer J R, Burns A R, Wilson B S, Oliver J M, Lidke D S 2008 Nat. Cell Biol. 10 955–63
[50] Andrews N L, Pfeiffer J R, Martinez A M, Haaland D M, Burger A S, Harding A S and Hancock J F 2008 Nature 451 314–20
[51] Cebecauer M, Spitaler M, Sergé A and Magee A I 2010 J. Cell Sci. 123 309–20
[52] Harding A S and Hancock J F 2008 Trends Cell Biol. 18 364–71
[53] Tian T, Harding A, Inder K, Plowman S, Parton R G and Hancock J F 2007 Nat. Cell Biol. 9 905–14
