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Review

Lipid–protein interactions in biological membranes: A dynamic perspective ☆

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ABSTRACT

Though an increasing number of biological functions at the membrane are attributed to direct associations between lipid head groups and protein side chains or lipid protein hydrophobic attractive forces, surprisingly limited information is available about the dynamics of these interactions. The static in vitro representation provided by membrane protein structures, including very insightful lipid–protein binding geometries, still fails to recapitulate the dynamic behavior characteristic of lipid membranes. Experimental measures of the interaction time of lipid–protein association are very rare, and have only provided order-of-magnitude estimates in an extremely limited number of systems. In this review, a brief outline of the experimental approaches taken in this area to date is given. The bulk of the review will focus on two methods that are promising techniques for measuring lipid–protein interactions: time-resolved fluorescence microscopy, and two-dimensional infrared (2D IR) spectroscopy. Time-resolved fluorescence microscopy is the name given to a sophisticated toolbox of measurements taken using pulsed laser excitation and time-correlated single photon counting (TCSPC). With this technique the dynamics of interaction can be measured on the time scale of nanoseconds to milliseconds. 2D IR is a femtosecond nonlinear spectroscopy that can resolve vibrational coupling between lipids and proteins at molecular-scale distances and at time scales from femtoseconds to picoseconds. These two methods are poised to make significant advances in our understanding of the dynamic properties of biological membranes. This article is part of a Special Issue entitled: Membrane protein structure and function.

Contents

1. Introduction

Biological membranes are complex, two-dimensional fluids formed from amphipathic lipid bilayers and a high density of proteins and carbohydrates. The organization of these components in live cells is heterogeneous, with order observed on a range of time and length scales. At a molecular level, it is the interaction potential between specific lipids and proteins that drives this organization, and to properly describe these interactions it is essential to understand the structure and dynamics of lipid–protein complexes. This has to some extent been observed using structural biology methods, where there are a growing number of membrane protein crystal structures in which a bound lipid has been identified [1]. These crystallized lipid–protein complexes are thought to be representative of strong, long-

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lived lipid–protein interactions in the plasma membrane. In other systems, lipids exchange rapidly from sites along the perimeter of a protein into the bulk two-dimensional fluid at a rate of $10^7\ s^{-1}$ [2]. These two cases represent the range of dynamics that govern lipid protein interactions, but there is still a vast unexplored field of systems for which these dynamics have not yet been measured. One reason for this lack of data is that the interactions are very difficult to observe using traditional structural methods because of the nature of the lipid–protein environment and because of the fast dynamics of the interactions. This review will first present a few examples where direct lipid–protein interactions are central to biochemical events. It will then briefly discuss the various approaches taken to measure the interactions. Finally, it will highlight two promising methods that can resolve the specificity and dynamics of lipid–protein complexes at a range of time and length scales.

1.1. Voltage-gated ion channels

Ion channels regulate charge transport across the plasma membrane. Gating and activity in these channels depend on a balance of ion concentration and electrical gradients across the membrane. In voltage-gated potassium channels, a sensor domain on the outside of the channel induces structural rearrangements that open the pore and lower the energy cost for ions to cross through the membrane [3]. This voltage-gating has been studied extensively, and there is growing evidence that the gating mechanism is lipid dependent [4–6]. For example, inward rectifier potassium channels are thought to directly bind negatively charged $PI(4,5)P_2$ lipids, and that the channel activity is proportional to the $PI(4,5)P_2$ concentration [6]. It has also been found that KvAP channels reconstituted in a bilayer with cationic lipids lacking a phosphodiester group lose their voltage-gated behavior [4]. When phospholipids are added to the bilayer, the channels recover their voltage-gated activity with the largest enhancement resulting from anionic phospholipids. The hypothesized mechanism is that positively charged arginine side chains form hydrogen bonds with negatively charged phosphate groups in the lipid [5,7]. This is consistent with lipid composition variations and directed point mutations [8], but to date there has been no direct probe of specific lipid head group–protein side chain interactions. Anionic phospholipids are also thought to regulate the activity of the mechanosensitive ion channels like MscL [9]. It has been found that increasing concentrations of phosphatidylglycerol, phosphatidic acid, or cardiolipin increase calcein flux through the MscL channel [10], and that the effect is likely due to direct hydrogen bond formation rather than differences in spontaneous curvature [11].

1.2. Lipid annulus of G protein-coupled receptors

G-protein coupled receptors are seven-transmembrane-helix proteins that participate in numerous signal transduction pathways. They play an essential role in vision, brain function and locomotion, and are the targets of over half of pharmaceutical treatments. The function of several GPCRs is cholesterol dependent [12–15], but one unanswered question is whether the interactions are specific, involving tight binding to well-defined protein regions, or if the effect is through physical changes to the surrounding membrane [16]. To ask this structural question is to ask about the time-scale and geometry of the cholesterol GPCR interactions. Cholesterol has been observed in crystal structures of rhodopsin and the β2 adrenergic receptor [13,17,18]. In the case of the β2 adrenergic receptor there is evidence of a cholesterol recognition amino acid consensus (CRAC) motif [18], which is a proposed cholesterol docking site [19]. It may be that these cases are unique, and that the larger dependence of GPCR function on cholesterol can be attributed to its modulating the physical properties of the membrane. However, it is clear from the literature that there is no consensus on the composition of the lipids adjacent to the protein and their respective time-scale of interaction.

Molecular dynamics simulations have been used to approach this problem for the nicotinic acetylcholine receptor (nAChR) [20]. The function of nAChR is dependent on the presence of the anionic phospholipid, phosphatidic acid (PA) [21], and is stabilized by cholesterol [22]. The simulations found that for lipids within 1 nm of the protein, PC and PA bilayers have the same thickness and order-parameter value, but that the PA lipids form a stable micro-domain around the protein [20]. This is evidence that the time-scale of lipid–protein interactions plays a key role in protein function, and argues for the development of new experimental approaches to directly test these hypotheses.

1.3. Lipid-binding domains

Integral membrane proteins are not the only class of proteins that interact specifically with membrane lipids. Cytosolic proteins make use of a variety of structural motifs to anchor them to lipid membranes [23,24]. For example, some proteins have amino acid sequences that encode for enzymatic attachment of lipid moieties that target the protein to a lipid bilayer [24,25]. These are often used in signaling pathways to regulate localization of the protein to the plasma membrane [24,25], and have also been proposed to localize the protein to specific functional membrane domains [26–28]. In spite of these observations, the physical interactions and the dynamic associations of these lipidated proteins are still poorly understood. For example, the lymphocyte cell kinase (Lck) protein is anchored to the membrane via two palmitoyl and a single myristoyl lipid modifications that are thought to target the protein to cholesterol-rich membrane regions [29,30]. However, there is evidence from biophysical studies that Lck partitions heterogeneously fluid domains in giant plasma membrane vesicles [26], which leaves open questions about the physical forces driving the organization of these proteins.

Another structural motif used to bind proteins to biological membranes is the inherent secondary and tertiary structures of the protein [23]. Such lipid binding structural motifs include target-specific domains like the C1 domain that binds diacylglycerol, the pleckstrin homology (PH) domain that binds phosphoinositides like PIP_2 and PIP_3, and FYVE domains that bind PIP_3. Other domains like PKC C2, annexin, BAR and F-BAR bind to anionic phospholipids non-specifically. While there is growing understanding of the protein–lipid binding kinetics, there are still core questions about secondary interactions. For example, PH domain binding to phosphoinositides is strengthened by insertion of hydrophobic amino acids into the bilayer as well as non-specific interactions with negatively charged lipids [23]. These interactions can be thought of as a two-dimensional analogue of co-solvent effects long studied in aqueous solutions. Central to the debate is how charge–charge interactions manifest themselves at the protein–lipid interface. Do anionic phospholipid head groups bind specifically to positively charged amino acid side chains, or is it better described as a non-specific co-solvent effect? Questions like this mirror those asked about the interaction of salts and osmolytes with proteins in aqueous solution [31]. To date, however, there has been almost no direct investigation of this effect in biological membranes.

2. Spectroscopic probes of lipid–protein interactions

Direct measures of lipid–protein interactions are difficult to make under physiologically relevant conditions. High resolution structural methods have begun to yield substantial insight. For example, several protein crystal structures have identified bound lipid molecules [1], including cholesterol bound to a GPCR as cited above [13,17,18], and a phorbol ester (a diacylglycerol analogue) bound to a C1 domain [32]. These crystal structures provide evidence for strong lipid–protein

interactions and the possibility of candidate sites for lipid binding. They do not, however, give any real measure of the interaction in native biological membranes, and they also cannot determine interaction dynamics and correlated structural fluctuations. Another difficulty is that those crystal structures represent only a fraction of possible lipid–protein interactions. Crystallography cannot be used in a majority of the systems of interest, where the interactions are comparably weak.

It is therefore desirable to use spectroscopic probes that are compatible with phospholipid bilayers at or near physiological temperatures [33]. NMR spectroscopy can be used to measure either lipid structure or protein structure, but has substantial difficulty in resolving intra-molecular coupling between lipids and proteins. As a result, there are few measurements of direct nuclear coupling between lipids and proteins [34,35]. One recent example was the observation of magnetization transfer from rhodopsin to the lipid acyl chain in reconstituted proteoliposomes [35]. The authors found that the rate of magnetization was highest for PE lipids and lowest with PC lipids, demonstrating that there are lipid specific binding sites on the rhodopsin protein [35]. Such studies, however, are rare and rely on long-lived interaction times.

Perhaps the most extensively used tool for measuring lipid–protein interaction dynamics is electron paramagnetic resonance (EPR) spectroscopy [36,37]. EPR measures the magnetic moment of unpaired electrons, which are typically introduced to lipids synthetically via a nitroxyl group and provide an environment-sensitive probe. Specifically, spin-labeled lipids near the protein–lipid boundary show a red-shift that is similar to EPR spectra of lipids at low temperatures. In this way protein-associated lipids have a unique spectroscopic signature that can be used to quantify the strength and time-scale of interaction. Spin–spin interactions can also be observed between different lipid species or between lipids and spin-labeled proteins. EPR has provided the bulk of evidence for the time scale of lipid–protein interactions including the reported 10^{-7} ns dwell time of lipids around the protein annulus [2]. The drawback of the method is that it is extremely difficult to transfer to live cells. This is mainly because EPR relies on synthetically attached probes, which also make it difficult to label large, multi-domain proteins and may perturb the details of the molecular interactions.

Optical spectroscopy overcomes the difficulties inherent to crystallography or NMR because of its faster time scales. It can also be performed without the use of extrinsic labels necessary for EPR. The increased dynamic resolution is often gained at the expense of structural resolution, but this can be overcome by selecting or designing a spectroscopically detectible degree of freedom with a clear structural interpretation. One traditional example of this is a fluorescence quenching assay in which tryptophan fluorescence quenching is observed when lipids such as cholesterol are in tight registry with the protein. This relies on tryptophan being natively incorporated into the protein at the site of interest, but once this is accomplished the interactions can be quantified with high precision in a room-temperature, aqueous environment.

The remainder of the review will deal directly with two emerging methods for measuring correlated, time-evolving structure of the lipid–protein interface: time-resolved fluorescence microscopy, and two-dimensional infrared spectroscopy (2D IR).

3. Time-resolved fluorescence microscopy

Fluorescence microscopy has long been a useful probe of biomolecules because of its high specificity and its compatibility with living samples. The last decade has seen significant advances in methodology, device engineering, and fluorescent probes, which have opened new possibilities for the study of lipid–protein interactions. For example, wide-field imaging of single molecules combined with stochastic photoactivation has been used to develop a suite of super-resolution microscopy methods [38–41]. These methods, as currently reported, are difficult to apply directly to lipid–protein interaction dynamics because of the lack of photoactivatable fluorescent lipids of interest, and because it would be difficult to arrest lipid diffusion without significant chemical perturbations. One approach that has been taken recently is to observe fluorescently labeled single protein molecules on a supported lipid bilayer (SLB). In one example, the guanine exchange factor GRP1 was imaged on supported lipid bilayers [42]. GRP1 has a PH domain that binds selectively to PI(3,4,5)P_3. By analyzing single molecule trajectories, the researchers observed that the diffusion coefficient of GRP1 bound to PI(3,4,5)P_2 was nearly identical to that of free lipids. When the anionic phospholipid, PS, was added to the bilayer, the diffusion coefficient decreased and the trajectories lengthened, indicating longer dissociation kinetics [42]. While such studies do not measure correlated motions of lipids and proteins, they still provide valuable insight into the membrane-specific protein dynamics especially when coupled with molecular dynamics simulations [43,44].

Fluorescence correlation spectroscopy is a single point excitation method that can be used to measure correlated motions of lipids and proteins [45,46]. FCS measures the fluctuations in fluorescence intensity, which at small volumes and low concentrations are dominated by the diffusion of fluorophores in and out of the excitation volume [45]. It is therefore sensitive to the number and diffusivity of the molecules of interest. Methods such as z-scan FCS [47], scanning FCS [48], and size dependent FCS [49] have all contributed more accurate measurements of the diffusion dynamics, and offer the potential to better understand lipid dynamics in synthetic bilayers and in live cells. Dual-color fluorescence cross-correlation spectroscopy (dc-FCCS) is a valuable FCS variant that is used to describe the interaction between the two fluorophore populations [50]. It quantifies the correlated diffusion of two populations of molecules over multiple time scales (~10^{-7} to 10^{1} s), and can measure the binding stoichiometry in simple systems. This method has been used primarily to determine protein–protein interactions [51], but can in principle be used to measure the lipid–protein dynamics where the fluorescent labels are chosen judiciously.

One challenge of dc-FCCS is spectral bleed-through, which leads to crosstalk between the two photodetectors and can give rise to inaccurate measures of population and overestimates the extent of cross-correlation. This has been overcome recently by using pulsed interleaved excitation (PIE) to bin photons according to the detector channel and the laser pulse present (Fig. 1 and Reference [52]). The pulses are spaced several multiples of the fluorescent lifetime so that each photon can be uniquely assigned to one of the two excitation lasers. In this way, photons arising from spectral crosstalk can be eliminated from the data processing, leading to very accurate measurements of molecular populations and correlated diffusion. A further advantage of PIE-FCCS is that it is collected in time-correlated single photon counting (TCSPC) mode. This means that the same photon data can be used to measure fluorescent lifetime histograms and photon counting histograms, which measure resonant energy transfer (i.e. FRET) and oligomer distributions respectively.

An integrated PIE-FCCS microscope is not commercially available and requires assembly or significant modifications to a commercial confocal microscope. Nevertheless, once the equipment is assembled, the technique can be applied to the same range of systems studied with conventional fluorescence microscopy. At one extreme, PIE-FCCS can be used to make high accuracy measurements of time-correlated lipid–protein dynamics in model systems like unilamellar lipid vesicles and supported lipid bilayers. At the other extreme, it can be used to measure lipid–protein interactions in heterogeneous systems like live cell membranes. In forthcoming work, PIE-FCCS is used to quantify the interaction of lipid-anchored protein constructs with membrane protein clusters in live cells, where it was found that the clustering is specific to the identity of the lipid moiety [53].

While dc-FCCS has been used to probe the preferential association of proteins with lipid domains [54–56], it has not been applied to

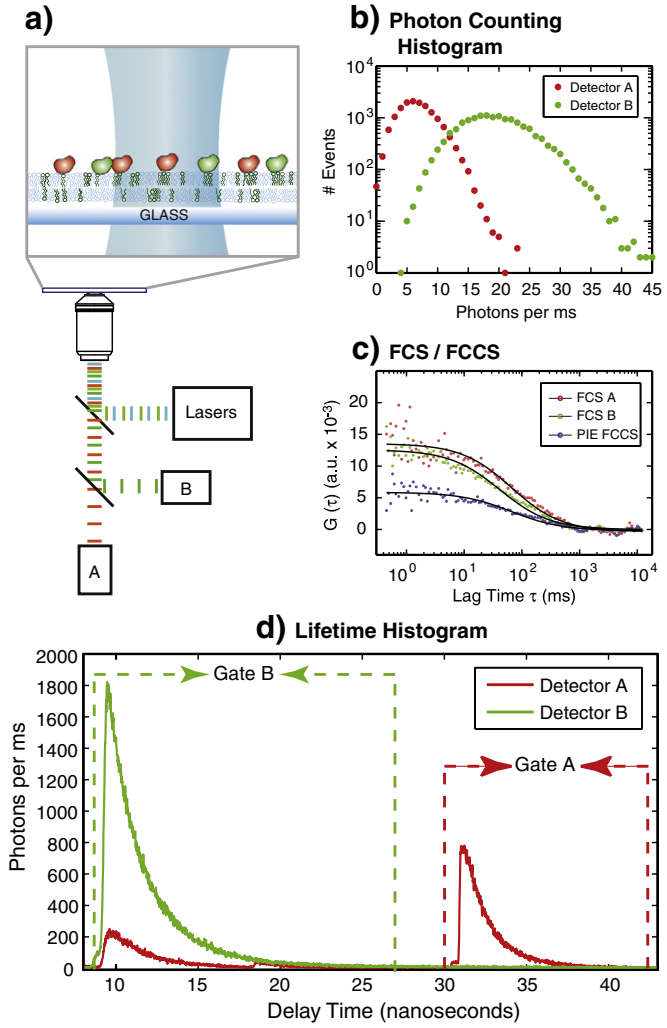

Fig. 1. Experimental diagram and sample data for a PIE-FCCS experiment. Diagram (a) shows a schematic of the experiment, which consists of two pulsed lasers at wavelengths designed to excite a red and a green fluorophore. The pulses are interleaved with a spacing dictated by the lifetime of the fluorophores. The emitted photons are split with a dichroic beamsplitter onto detector A (red fluorophore) and B (green fluorophore). The stream of photons can be used to calculate (b) a photon counting histogram to measure brightness and fluorophore densities, (c) FCS/FCCS spectra to measure diffusion times and co-diffusion, and (d) lifetime histograms to probe the environment of the sample and measure resonant energy transfer (FRET). With the interleaved pulses, artifacts can be removed by time-gating the photons to remove red fluorophore bleedthrough to the green detection channel.

study correlated motions of lipids and proteins. This is potentially the greatest strength of dc-FCCS and has yet to be fully realized. Because dc-FCCS has the ability to resolve correlated diffusion, it could reveal the number of lipids that co-diffuse with the protein. This would be of tremendous value in the biological systems cited in the Introduction and would provide evidence for (or against) hypothesized diffusion complexes that have been observed in molecular dynamics simulations [57]. Slaved diffusion has already been observed for lipid bilayers with electrostatically bound polyelectrolytes [58], but it is not known whether the electrolytes drag around a shell of lipids or whether the long range lipid motions are statistically uncorrelated. Methods like dc-FCCS have the potential to make significant impact in this area.

4. 2D IR spectroscopy

Fluorescence measurements require attaching fluorescent probes that are on the same size scale as the lipid itself and will have an effect on the interaction between the protein and lipid. This is less of a problem for protein–protein interactions, where fluorescent probes can be designed at locations that do not disrupt the functional interface. While it is possible in a few systems to attach a fluorescent probe to the lipid in a non-perturbative way, it would be preferable to use a probe that does not require extrinsic labels. Time-resolved infrared spectroscopy has the potential to directly access the protein–lipid interface without labeling or with isotope labels that do not significantly perturb the molecular interaction energies. 2D IR spectroscopy is a well-developed probe of molecular structure and dynamics. Analogous to multi-dimensional NMR, 2D IR spectroscopy is a pulsed technique that excites vibrational coherences and populations at time-scales comparable to the vibrational period (Fig. 2). Peaks along the diagonal axis of the 2D IR spectrum correspond to the linear absorption spectrum, while off-diagonal cross peaks correspond to coupling between vibrational modes.

2D IR spectroscopy is a femtosecond, nonlinear optical method that is not commercially available as an integrated instrument. It is, however, growing in its use and range of applications, and can be found in an increasing number of labs worldwide. As a vibrational spectroscopy, 2D IR is subject to spectral congestion and is currently limited to simple systems with low numbers of unique components. While a $\sqrt{2}$ resolution enhancement is gained by spreading the spectrum into a second dimension, it is often necessary to use isotope labels to isolate resonances of interest. A typical concentration for 2D IR spectroscopy is around 1 mM [59], but can be lower especially if there is more than one vibrational chromophore per molecule. This concentration is high for many aqueous proteins, but matches the molarity of lipids in typical vesicle suspensions [60].

2D IR spectroscopy has been used in a variety of applications including chemical exchange in small molecules, solvent hydrogen bonding dynamics, and protein structure and dynamics [61–64]. While primarily used to study aqueous proteins and peptides, 2D IR has also been used to probe the structure of lipids and membrane proteins. For example, 2D IR spectroscopy was used to measure the

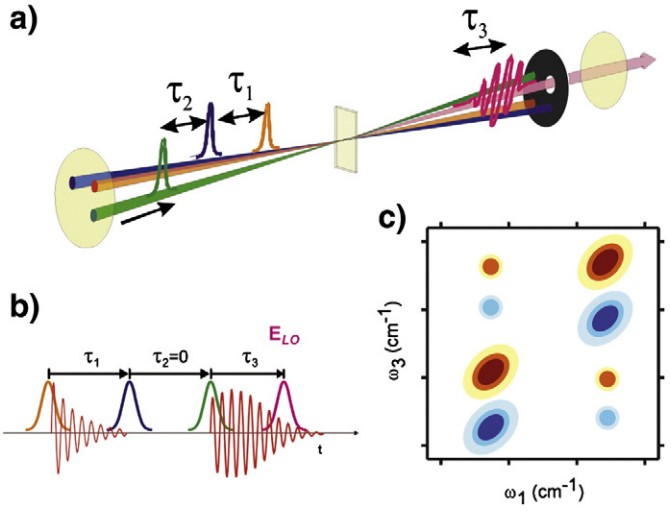

Fig. 2. Two-dimensional infrared (2D IR) spectroscopy is a nonlinear technique that uses a series of infrared pulses to probe the sample. Diagram (a) shows the experimental geometry of the three excitation beams and nonlinear signal beam overlapped with a local oscillator field for heterodyne detection. Panel (b) shows the pulse timing diagram to illustrate that the 2D IR signal is obtained by scanning time delays τ_1 and τ_3. The 2D IR spectrum is produced by a two-dimensional Fourier transform of the τ_1 and τ_3 axes at a fixed τ_2. (c) The 2D IR spectrum shows pairs of positive (red) and negative (blue) peaks that reflect the fundamental and overtone vibrational transitions respectively. Diagonal peaks are related to the absorption peaks in an FTIR spectrum, and cross peaks indicate coupling between vibrational modes. The shapes of the peaks also contain information about static heterogeneity at picosecond time scales.

vibrational spectrum of carbonyl moieties in dimyristoyl-phosphotidylcholine (DMPC), where cross peaks and line shape analysis were used to quantify intermolecular electrostatic coupling [65]. In more recent work by the same lab, the incorporation of a myrisoylated glycine (MrG) dipeptide into a 1-palmitoyl-2-linoleyl phosphatidylcholine (PLPC) bilayer was studied with 2D IR spectroscopy [66]. This is a highly relevant model system to understand the molecular details of lipid-anchored protein binding to biological membranes. The work found that the lipid-anchored peptide primarily occupies an unfolded conformation ($\Phi = -100°$, $\Psi = 180°$) where the peptide backbone is oriented 60° with respect to the plane of the bilayer. The peptide carbonyl vibrations are dominated by homogeneous broadening, indicating very little conformational heterogeneity in contrast to small peptides in solution. Finally, the 2D IR cross peaks between the peptide carbonyls and the PLPC carbonyl moieties were used to show that MrG is strongly associated with the phospholipids, and that the angle between the respective carbonyl groups varies between 20° and 50° [66].

Larger membrane-associated proteins and peptides have also been studied with 2D IR spectroscopy [67–71]. For example, the 2D IR lineshape of amide groups exposed to water in the M2 proton channel pore shows increased diagonal line-width compared to those buried in the bilayer hydrophobic core [67,68]. A systematic set of isotope labels showed that the channel exposed amino acids at low pH matched the geometry of the open channel based on structural biology data. At high pH, in the closed channel conformation, the pattern of line-shapes revealed a concerted, one amino acid shift in the α-helix registry [68]. This previously unresolved structural transition demonstrates the utility of 2D IR spectroscopy to study membrane protein structure in situ, where the effects of the environment exert significant influence on biologically relevant conformational dynamics.

The ability of 2D IR spectroscopy to resolve lipid–protein interactions has been demonstrated in the case of the MrG–PLPC system described above [66]. It has also been shown for a membrane spanning peptide, CD3ζ, in DMPC vesicles, where a lipid–peptide cross peak was observed [59,72]. The 2D IR cross peak indicates significant vibrational coupling, and shows that nonlinear infrared spectroscopy is a useful tool for investigating the dynamics of lipid–protein interactions. One approach that has not been taken is to measure coupling between the lipid headgroup and positively-charged amino acid side chains like arginine. This would directly test models for lipid-mediated protein function, as in the case of voltage-gated potassium channels, and would provide a way to measure the effects of solvent or lipid composition. The dynamics of the interface could also be probed with a variety of pulse delay experiments to measure the lifetime of the vibrational energy transfer. In addition, the cross peak could be observed for various lipid compositional variations to probe how the strength of the interaction is modified by specific lipid head groups.

5. Conclusion

As our knowledge of the complexity of the plasma membrane grows, so does the need to develop methods to probe structure and dynamics at a range of time and length scales. To this end, there is tremendous value in emerging methods that directly measure protein–lipid interactions with molecular-scale sensitivity and fast time resolution. This review has focused on two methods that have undergone significant developments in recent years: time-resolved fluorescence microscopy and 2D IR spectroscopy. Two-color fluorescence correlation spectroscopies can directly probe correlated diffusion of lipids and proteins at length scales determined by the size of the observation area and time scales determined by the diffusion time of the molecules of interest. For diffraction limited techniques, this translates to areas near 0.2 μm^2 and time scales from 10^{-7} to 10^{1} s. The resolution can be improved with recently developed STED-FCS, which can achieve areas as small as 0.005 μm^2 [73], and offers tremendous promise for the study of plasma membrane dynamics. 2D IR spectroscopy probes vibrational resonances and coherence on the femtosecond to picosecond time scale. Lipid–protein vibrational energy transfer has already been observed in a number of systems, and can be used to resolve outstanding questions in protein dynamics within biological membranes.

These experiments have the potential to provide direct evidence to test hypotheses regarding lipid–protein interactions that have been made extensively in the literature. They will also go hand in hand with molecular dynamics simulations of biological membrane systems to focus on specific molecular interactions [74]. In this way, it will be possible to enhance our knowledge of the membrane beyond well-developed continuum theories to build a comprehensive physical description of organization and dynamics in live cell membranes.

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Further reading

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