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Investigations of membrane protein interactions in cells using fluorescence microscopy

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Abstract

The interactions between proteins in membranes govern many cellular functions. Our ability to probe for such interactions has greatly evolved in recent years due to the introduction of new fluorescence techniques. As a result, we currently have a choice of methods that can be used to assess the spatial distribution of a membrane protein, its association state, and the thermodynamic stability of the oligomers in the native milieu. These biophysical measurements have revealed new insights into important biological processes in cellular membranes.

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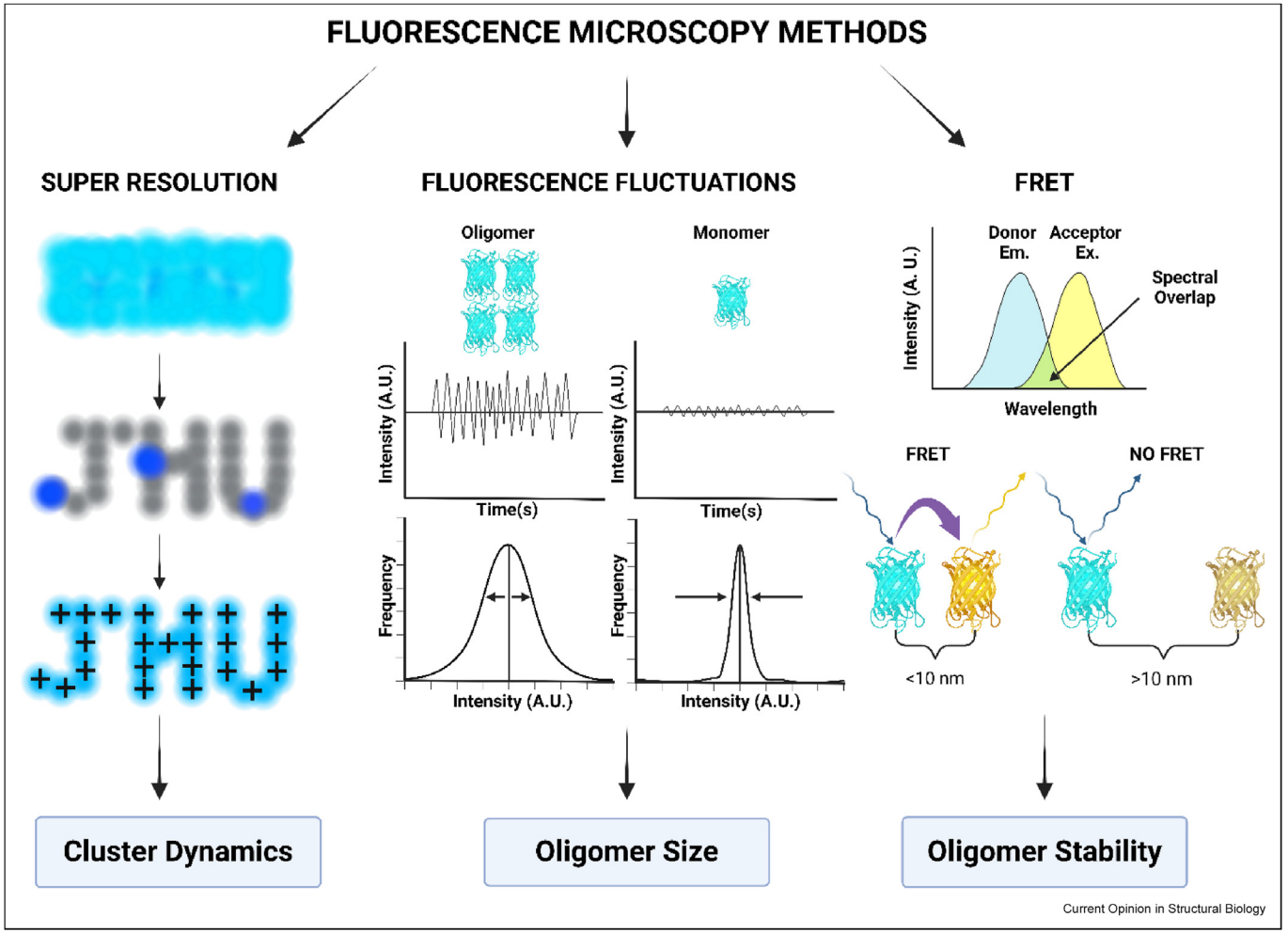
Introduction

Membrane proteins are abundant in eukaryotic cells, comprising an estimated 30% of all genes [1]. They are key to many cellular functions including signaling, cell adhesion, motility, metabolism, and flux of metabolites through cells [2,3] and are thus subjects of intense scientific investigations. For many years, however, progress in membrane protein research has been relatively slow, especially when compared to studies of soluble proteins [4,5]. This is now rapidly changing, and our mechanistic understanding of membrane protein structure-function relationships is steadily growing [6].

The function of membrane proteins is often controlled by their association state, in homo and heterocomplexes. One of the best-known examples is the family of receptor tyrosine kinases (RTKs), which are single-pass membrane proteins that are inactive when monomeric but become active upon lateral association into dimers or higher order oligomers. Within the oligomeric complexes, the kinase domains are in close proximity, where they cross-phosphorylate each other and initiate downstream signaling. As a result, RTK function can be tuned through changes in RTK lateral association [7]. For other membrane proteins, the functional effects due to protein-protein interactions can be more subtle but nevertheless highly significant. For instance, the seven transmembrane helix G protein—coupled receptors (GPCRs) can signal as monomers while undergoing conformational changes in response to their ligands. Lateral interactions between GPCRs can impact the ligand-induced conformational changes and alter GPCR signaling [8—10]. In addition, membrane receptors can organize themselves into spatially enriched domains, leading to heterogeneous spatial distributions in the membrane. Within the enriched domains, the receptors may engage in either direct receptor-receptor interactions or indirect interactions that are mediated by lipids or cytoplasmic proteins [11]. The structural features of the enriched domains (also referred to as clusters) are not well understood, but there is evidence of differential functioning of the receptors inside and outside the domains [12].

Recent work has provided many examples of membrane protein interactions in cellular membranes that have functional significance [13—15]. Studies which utilize fluorescence have been instrumental in identifying and quantifying these interactions as they allow us to interrogate the dynamics of membrane proteins in the context of the native cellular environment [16]. Here, we overview recent experimental studies of membrane protein interactions that rely on three broad classes of fluorescence methods: super resolution microscopy, fluorescence fluctuation techniques, and Förster Resonance Energy Transfer (FRET) (Figure 1).

Figure 1



A schematic of the discussed fluorescence microscopy techniques used to study membrane protein interactions. Super-resolution techniques resolve individual fluorophores to construct an image with much higher resolution than what can be achieved using conventional microscopy. Such techniques can shed light on the presence and dynamics of membrane protein clusters in cells. Fluctuations of fluorescence intensities over time or space can be quantified to determine membrane protein oligomer sizes. FRET techniques utilize pairs of fluorophores (donors and acceptors) to report on close molecular distances. When donor and acceptor fluorophore pairs are within sufficient proximity, a spontaneous energy transfer occurs in which the emission of the excited donor excites the acceptor molecule. FRET techniques are highly sensitive to changes in molecular distances (within nanometers) as the efficiency of this energy transfer is correlated to the distance between the pairs. Protocols have been developed to extract dissociation constants that report on the stability of oligomers of membrane proteins [17].
Abbreviation: FRET = Förster Resonance Energy Transfer.

Super-resolution methods

Super-resolution broadly encompasses a series of techniques that aim to bypass the traditional diffraction-limited resolution of light in microscope setups. These techniques involve either deterministic analysis of differential fluorophore responses to excitation or stochastic activation and reconstruction of individual fluorophores [18–21]. Deterministic super-resolution, such as stimulated emission depletion (STED) microscopy, focuses on utilizing the nonlinear emission response of fluorophores. Alternatively, single-molecule localization microscopy (SMLM), which includes photoactivated localization microcopy, encompasses a set of methods that utilize on/off properties of fluorophores that allow them to be resolved over time [22]. Super-resolution techniques have been reviewed in detail recently [23–25], and the readers are referred to these reviews for a comprehensive discussion of the different methodologies. Over the last decade, super-resolution microscopy has yielded new insights into membrane protein association. It has uncovered patterns of spatial organization and regulatory mechanisms and has allowed researchers to probe for membrane protein biological function in the native cellular milieu.

Increasingly, super-resolution techniques are used to investigate clustering of molecules and to characterize changes in spatial distributions of membrane proteins in response to stimuli. These properties may begin to address how the spatial distribution of membrane proteins affects their function. In one example, researchers utilized direct stochastic optical reconstruction microscopy, an SMLM technique involving stochastic activation of conventional fluorophores, to visualize the distribution of the glucagon receptor (GCGR), a member of the class B G protein–coupled receptor family, in the plasma membrane of HepG2 (human-liver cancer) cells [26]. GCGR plays an important role in the regulation of the glucose levels as its ligand glucagon initiates GCGR-mediated signaling cascades that lead to the conversion of glycogen to glucose. In a feedback loop, extracellular glucose levels regulate the expression of GCGR in the plasma membrane. The super-resolution experiments revealed the presence of nanoscale-sized clusters in the membrane, with larger clusters formed in response to higher glucose concentration. At low glucose concentrations, treatment with glucagon decreased GCGR expression and the cluster size. At high glucose concentrations, however, glucagon had much smaller effects on the expression and the cluster size as the large and dense clusters of GCGR persisted despite glucagon addition. These studies therefore revealed different GCGR organizations at different glucose levels and showed that GCGR is resistant to the effects of glucagon under high glucose conditions. The organization of GCCR in the membrane impacts processes that underlie diabetes, giving hope that novel strategies that target the membrane organization could be developed in the future to combat dysregulation of glucose levels in patients.

Another prominent application of super-resolution microscopy involves the use of dual colors to shed light on heterointeractions and colocalization of membrane proteins and to validate existing biochemical data. A recent study investigated the membrane distributions of two RTKs, cMET and EGFR, which play important roles in cell motility, differentiation, and growth, and in the induction of non-small-cell lung carcinoma (NSCLC) [27]. The heterointeractions between cMET and EGFR were investigated using exchange-PAINT, a multiplexed imaging approach that yields single-molecule localization through the transient binding events of short oligonucleotides labeled with a fluorophore to a complementary oligonucleotide conjugated to an antibody against a membrane protein domain. Experiments were conducted that visualized and quantified clusters of cMET/EGFR in response to ligand stimulation in two cancer cell lines with distinct expression levels of the receptors [27]. The analysis revealed that cMET and EGFR colocalization increased upon addition of ligands in both HeLa and BT-20 cancer cells. We envision that these findings may prove instrumental to the design of therapeutics that simultaneously target two different RTKs in NSCLC and other RTK-related cancers. The overviewed study of the dynamics of EGFR and cMET is particularly exciting as a bispecific antibody-drug conjugate (AZD9592) has recently been developed to target both EGFR and cMET and has shown significant therapeutic potential in NSCLC patient–derived xenograft models [28]. An expansion of the super-resolution microscopy study that includes AZD9592 can provide insights into the mechanism of action of the novel drug.

Super-resolution microscopy continues to see developments that expand the toolbox for membrane protein studies, for instance, allowing for three-dimensional topographic investigations of membranes at high resolution. A notable recent study utilized three-dimensional STED microscopy to investigate the mechanism by which GPCRs organize into domains in the plasma membrane [29]. This study is particularly significant, given that the authors were able to visualize the membrane topography and the curvature, as well as the density of the receptors, a nontrivial technical challenge in the field. Through this strategy, the authors could more accurately relate how changes in membrane topology and receptor organization are coupled. These investigations revealed that the plasma membrane curvature plays an important regulatory role in GPCR domain formation and can impact GPCR function. A

potential organizing principle for membrane proteins emerged through these experiments as the observed curvature-based segregation behavior was shared by multiple receptors, not just GPCRs. Based on this work, it was proposed that targeting the receptor preference for a specific curvature may be a novel way to modulate receptor distribution and, therefore, receptor function. We envision that super-resolution microscopy will continue to provide much-needed views of the organization of membrane proteins as a function of the topology of the membrane they reside in.

Fluorescence fluctuations methods

Proteins in the membrane move randomly in time and space due to Brownian motion. If the proteins are tagged with fluorophores, these motions can be visualized in fluorescence correlation spectrometry (FCS) experiments [30−32], which follow fluorescence fluctuations over time and/or over space. The fluorescence intensities of spatially dispersed monomers or an oligomer can be the same, but fluctuations in fluorescence intensity of higher order oligomers are larger because all proteins in the oligomers codiffuse together in and out of a pixel. FCS experiments can be analyzed using an autocorrelation function that reports on the timescale of the fluctuations and can yield information about the dynamics of membrane proteins, allowing researchers to deduce the oligomerization states of membrane proteins [33].

Different variations of FCS have been developed, including fluorescence cross-correlation spectroscopy to study heterointeractions [34]. Raster scanning image correlation spectroscopy (RICS) is a correlation method that allows the analysis of both temporal and spatial intensity fluctuations while scanning a laser beam over a sample [35]. In the recent years, RICS has been used to determine the diffusion coefficients of the membrane-associated cluster of differentiation 14 (mCD14) and Toll-like receptor 2 (TLR2), which are proteins found in the plasma membrane of macrophages [36]. TLR2 is a single-pass type I membrane protein involved in the activation of the innate immune system, whereas mCD14 is a membrane-anchored one, via a glycosylphosphatidylinositol tail, glycoprotein, which acts as a co-receptor essential for TLR2 activation [37]. Careful measurements of the diffusion constants of these receptors revealed that mCD14 diffusion was significantly faster than the diffusion of TLR2. The authors proposed that the absence of a cytoplasmic domain in mCD14 might account for the faster lateral movement, which may be needed for rapid ligand binding. The bound ligand can be then delivered to TLR2, to activate it more efficiently, thereby suggesting a regulatory mechanism for TLR2 activation.

In recent years, spatial fluctuation techniques such as spatial intensity distribution analysis (SpIDA) and fluorescence intensity fluctuations (FIFs) have been developed and used to study the association of membrane proteins into dimers and higher-order oligomers [38,39], without explicit calculations of diffusion constants. SpIDA calculates the so-called “brightness” values from the mean fluorescence intensity and variance in a selected region of interest (ROI) of the membrane. The brightness is proportional to the oligomer size. FIF can be considered an enhanced form of SpIDA as the selected ROI of the cell membrane is further segmented into submicron-sized tiles, and brightness values are calculated based on the fluorescence mean and variance in each tile and then histogrammed [38,39]. Milligan et al. [40] used both SpIDA and FIF to study the organization of the M1 muscarinic receptor, a GPCR involved in the regulation of the central nervous system. As an important development, the authors applied SpIDA and FIF to a neuronal culture derived from transgenic M1-mEGFP-expressing mice. They showed that the receptor population is heterogeneous, composed of monomers, dimers, and oligomers. The addition of both agonist and antagonist ligands lead to a significant increase in monomerization, suggesting that the oligomer size of the receptor may affect receptor function. These fluctuation techniques are expected to be crucial in the future. The possibility of performing measurements in various cell types, as in Ref. [40], will allow us to study cell-specific membrane processes with quantitative tools to reveal detailed mechanisms.

Number and brightness (N&B) is another fluctuation technique that has been used to assess the oligomer size of membrane proteins in the cells by measuring brightness in a time-resolved manner. N&B is sometimes preferred over FCS as the latter is only able to detect slow-diffusing molecules [41,42]. Enhanced N&B, a variation of N&B that performs data resampling, was introduced by Ojosnegros et al. [43]. The method was developed with the goal of understanding the oligomer size distributions of EphB2, an RTK involved in the regulation of the nervous system and implicated in pancreatic cancer, in response to the ligand ephrinB1. The authors followed the time course of EphB2 clustering upon ligand addition and were able to explain the measured time course based on the Lumry−Eyring theory of protein aggregation. They proposed that EphB2 operates through an on−off/polymerization−condensation mechanism. The authors further suggested that the dimer state promotes EphB2 activation and signaling, whereas the oligomeric states lead to EphB2 deactivation and endocytosis. This study represents one of the first quantitative studies of the dynamic behavior of the Eph receptors, which are known to invoke a myriad of signaling responses leading to diverse, and even opposite, cellular outcomes.

Paul et al. introduced a variation of N&B (cdN&B) in which the brightness was measured as a function of membrane protein concentration in the plasma membrane [44]. The data were fit to thermodynamic models of association, yielding association constants for several single-pass receptors. While most of the studied receptors were found to form dimers, the neuropilin 1 (NRP1) receptor was shown to form a higher order oligomer, most likely a tetramer. NRP1 is known to interact with multiple partners in the plasma membrane and is implicated in many cancers and neurological disorders. One possible hypothesis is that the self-association of NRP1 into homo-oligomers provides a mechanism for the receptor to sequester itself until its ligands and coreceptors become available. The understanding of the behavior of NRP1 is important for understanding angiogenesis, the growth of new blood vessels from preexisting ones [45]. This process is critical for the growth of solid tumors as they need oxygen. Angiogenesis is mainly controlled by the RTK VEGFR2 and its ligand VEGFA, which are known to both interact with NRP1 [46,47]. The details of these interactions are not well understood, despite their enormous physiological significance. The NRP1 cdN&B study sets the stage for comprehensive investigations of the VEGFR2/VEGFA/NRP1 signaling axis.

Förster Resonance Energy Transfer methods

While fluctuation techniques are well suited to measure the oligomer size of a membrane protein complex, FRET is best suited to measure dissociation constants that report on the stability of the membrane protein oligomers [48]. FRET occurs when donor- and acceptor-labeled proteins interact, which brings the donors and acceptors near each other. FRET is often referred to as a "molecular ruler" as the efficiency of energy transfer depends on the inverse sixth power of the distance between the donor and acceptor fluorophores [49]. The Förster distance, R_0, is influenced by several factors, most prominently the spectral properties of the donor and acceptor fluorophores. R_0 is $\sim$5 nm for most FRET pairs used in membrane protein interaction studies [50–52].

In quantitative FRET experiments, the expression of the membrane proteins is varied over a wide range, and membrane protein concentrations in the plasma membrane are measured in hundreds of cells, along with FRET efficiencies, yielding oligomerization curves. These experiments are not trivial, however, because FRET is not simply an oligomerization assay. Four different intertwined effects contribute to the measured FRET efficiencies in the experiments: oligomer size, oligomer stability (given by the dissociation constant), oligomer structure (i.e. distance between fluorescent proteins), and proximity FRET. Protocols such as the so-called "fully quantified spectral imaging (FSI)" have been developed to address and mitigate these effects [48]. By using two excitation wavelengths, this method allows the calculations of FRET efficiencies and donor/acceptor concentrations without assumptions about the spectral properties of the fluorophores. Additionally, fluorescence intensities are correlated with concentrations using donor and acceptor solution standards, surpassing the limitations of methods that rely on a single calibration standard imaged at two wavelengths [48]. The FSI-FRET was used to understand how the human herpes virus (HHV-8) hijacks the EphA2 receptor to not only enter cells but also promote oncogenic signaling that leads to Kaposi sarcoma [53]. EphA2 is an RTK that controls short-distance cell–cell communications, cytoskeleton rearrangements, and changes in cell morphology in response to its ligands, the ephrins. EphA2 dysregulated signaling has been linked to tumorigenesis and the progression of several cancers. The FRET studies revealed that the HHV-8 envelope glycoprotein complex H and L (gH/gL) binds to EphA2 to induce EphA2 dimerization, which in turn leads to EphA2 activation and cell contraction. Furthermore, gH/gL binding to the extracellular domain of EphA2 induces structural changes that propagate to the EphA2 kinase domain, which initiates signal transduction. These FRET studies shed light on the biological significance of the high-resolution structure of gH/gL in a complex with the ligand-binding domain of EphA2. They also reveal a link between the action of the virus and the engagement of a membrane receptor, which has been implicated in many cancers, hinting at the possibility that EphA2 may be contributing to the pathogenesis in Kaposi sarcoma caused by HHV-8.

Conclusions and perspectives

Our understanding of membrane protein organization and activation relies heavily on our technical capacity to study them. Membrane proteins have been historically challenging to study in purified form, but fluorescence has allowed us to investigate the dynamics of these proteins in their native environment without the need for extraction, purification, and reconstitution. During the last decades, fluorescence microscopy techniques have consistently proven to be among our most effective tools to observe membrane proteins within their physiologically relevant environments. Here, we overviewed some of the recent studies that use fluorescence techniques to gain insight into membrane protein interactions and self-organization. This is just a small peek into an important and fast-growing field.

Despite the utility of the overviewed methods, we recognize that no individual fluorescence technique is sufficient to yield a holistic view of membrane protein behavior. We hope that future investigations of membrane proteins will utilize multiple fluorescence methods, along with other biophysical and biochemical methods, to reveal models of membrane protein behaviors that are consistent with all the data. For example, cdN&B measurements that report on the average oligomer size can complement FRET experiments, which report on oligomer stability. Moreover, we look forward to the continuous refinement of available fluorescence tools and the development of new ones. We believe that techniques that provide both spatial and temporal information will be most useful. We also hope that future research in the field will shed light on how the membrane milieu exerts control over membrane proteins dynamics, organization, and function. Questions that can be pursued in the near future to reveal mechanistic information include the following:

- How do cells regulate membrane topology and how does that affect the activation of membrane receptors?
- How does lipid composition affect membrane protein organization and function?
- How does membrane surface glycosylation affect membrane protein dynamics?
- What role do biomolecular condensates play in membrane protein spatial distribution?

There is no doubt that the insights gained through fluorescence studies will be key in shaping our understanding of the action of membrane proteins in the years to come. We anticipate that these methods will continue to be applied to diverse classes of membrane proteins in the future. Furthermore, we expect that the basic knowledge gained through these studies will empower the search for novel approaches to modulate the function of membrane proteins. As membrane proteins are strongly implicated in many diseases including a variety of cancers, these efforts may eventually lead to the development of novel therapeutics with high specificity and low toxicity. Increasingly, knowledge regarding membrane receptor clustering and heterointeractions are used to design novel targeting molecules, such as antibody/protein drug conjugates, which can increase receptor internalization and improve the efficacy of therapeutics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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