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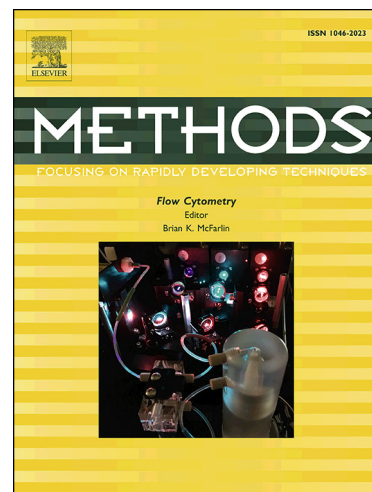
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## Single molecule fluorescence for membrane proteins

Oliver K. Castell<sup>a\*</sup>, Patricia M. Dijkman<sup>b</sup>, Daniel N. Wiseman<sup>c</sup>, Alan D. Goddard<sup>c\*</sup>

<sup>a</sup> School of Pharmacy and Pharmaceutical Sciences, College of Biomedical and Life Sciences, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK. [Castello@cardiff.ac.uk](mailto:Castello@cardiff.ac.uk)

<sup>b</sup> Max Planck Institute for Biophysics, Max-von-Laue-Straße 3, 60438 Frankfurt am Main, Germany. [patricia.dijkman@biophys.mpg.de](mailto:patricia.dijkman@biophys.mpg.de)

<sup>c</sup> School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK. [d.wiseman1@aston.ac.uk](mailto:d.wiseman1@aston.ac.uk)

\* Corresponding authors. [Castello@cardiff.ac.uk](mailto:Castello@cardiff.ac.uk) [a.goddard@aston.ac.uk](mailto:a.goddard@aston.ac.uk)

### Abstract

The cell membrane is a complex milieu of lipids and proteins. In order to understand the behaviour of individual molecules it is often desirable to examine them as purified components in *in vitro* systems. Here, we detail the creation and use of droplet interface bilayers (DIBs) which, when coupled to TIRF microscopy, can reveal spatiotemporal and kinetic information for individual membrane proteins. A number of steps are required including modification of the protein sequence to enable the incorporation of appropriate fluorescent labels, expression and purification of the membrane protein and subsequent labelling. Following creation of DIBs, proteins are spontaneously incorporated into the membrane where they can be imaged via conventional single molecule TIRF approaches. Using this strategy, in conjunction with step-wise photobleaching, FRET and / or single particle tracking, a host of parameters can be determined such as oligomerisation state and dynamic information. We discuss advantages and limitations of this system and offer guidance for successful implementation of these approaches.

### Keywords

Membrane protein, single molecule fluorescence, droplet interface bilayer, FRET, photobleaching, TIRF.

### Introduction (~600 words)

The cell membrane can be envisaged as a crowded lipid bilayer in which protein-protein and protein-lipid interactions are responsible for regulating membrane function including maintenance of cell structure, transport of molecules in and out of the cell and signal transduction. Historically, most biological analyses have been performed on populations using ensemble averages. However, cells represent heterogeneous environments where fluctuations in protein activity may be inherent to biological function. This is especially true in the membrane where the “solvent”, i.e. the lipids in the membrane, is itself heterogeneous and the presence of localised populations of proteins and/or microdomains could have significant impacts on protein function [1].

Single molecule techniques provide an incredibly powerful approach with which to probe membrane protein localisation, interactions and dynamics [2]. The strength of these approaches lies in the visualisation and analysis of individual molecules which can reveal information not accessible via ensemble techniques [3]. For example, single molecule analysis removes ensemble averaging and can therefore reveal, and be used to analyse, subpopulations of molecules. In addition, kinetic analysis can be performed on unsynchronised populations, enabling insight into the nature of dynamic equilibria. The ability to analyse heterogeneous populations can provide insights that would be masked in ensemble techniques [4], affording the opportunity to reveal spatio-temporal dynamics, rare-states, transient intermediates and population stoichiometries.

Single molecule fluorescence techniques have been applied to a range of both soluble and membrane proteins. In order to undertake these approaches, a number of technical challenges must be overcome. Firstly, unless a direct activity can be measured e.g, ion flux imaging the membrane protein of interest

must be modified in order to allow site-specific labelling with an appropriate fluorophore [4]. The chosen fluorescent molecule must have appropriate spectral properties, a particularly important consideration if techniques such as Förster resonance energy transfer (FRET) are to be employed. If the protein is to be studied in an artificial bilayer, it must be purified and reconstituted [5]. To achieve resolution of single particles, background noise must be overcome, which is often achieved through volume restriction and microscopy techniques such as total internal reflection fluorescence (TIRF) [6]. Finally, computational techniques must be applied in order to perform appropriate analysis of the sample [7].

There are a number of excellent reviews of single molecule fluorescence applications [8-10] covering a field which, in its entirety, is beyond the scope of this article. Traditionally, due to the combined challenges of maintaining proteins in a folded, functional state in a membrane-like environment and interfacing with suitable measurement techniques, membrane protein research has lagged behind that of soluble proteins, including the application of single molecule techniques. A number of systems have been developed to address these combined challenges and enable these analyses to take place. We will focus on Droplet Interface Bilayers (DIBs) [11], a versatile platform for *in vitro* membrane studies [12], providing a lipid bilayer environment into which transmembrane proteins can be reconstituted, diffuse freely and be imaged by single molecule techniques. These platforms also provide the ability to make single channel electrophysiology measurements and afford control of both membrane composition and chemical environment either side of the bilayer, creating versatile opportunities for the study of kinetics and association of purified, labelled, reconstituted membrane proteins in a well-controlled *in vitro* environment.

## **Imaging Membrane Proteins in DIBs**

### ***Generating the components***

#### **Fluorescence, fluorophores and labelled proteins**

Fluorescence is a physical process that occurs when a fluorophore absorbs a photon and subsequently emits this as a photon of longer wavelength. The shift in wavelength arises due to loss of energy from the photon via oscillation between the atomic orbitals. Each fluorophore will have both an excitation and emission spectrum that defines the optimum wavelength used to stimulate the fluorophore and to measure emission of the fluorescence. The difference between the excitation and emission maxima is known as the Stokes' shift and, generally, a large Stokes' shift is beneficial as it reduces inadvertent detection of the excitation wavelength in the emission channel. Fluorescence is a rapid process, even on the biological timescale, making it ideal for probing cellular and protein systems. Additionally, due to the cyclical nature of the fluorescence process, as long as the fluorophore is not irreversibly destroyed in the excited state (photobleaching), a single fluorescent molecule can generate thousands of photons allowing high sensitivity.

To perform single molecule fluorescence studies it goes without saying that the membrane protein of interest must be labelled with a suitable fluorophore. A number of key decisions must be made when selecting the fluorophore, especially considering the inherent intractability of membrane proteins. The main considerations are, firstly, the photophysical properties of the fluorophore i.e. its suitability for the single molecule technique of interest and, secondly, the ability to attach the label to the protein in a site-specific manner without perturbing protein behaviour [4].

For single molecule detection, it is vital to be able to detect a weak fluorescence emission signal against background noise. A key feature of the fluorophore is hence the brightness [13] which is proportional to the extinction coefficient and the quantum yield. The extinction coefficient is defined as the quantity of light absorbed at a particular wavelength and the quantum yield is defined as the number of photons emitted divided by the number absorbed. As such, high extinction coefficients and quantum yields approaching unity are desirable features of fluorophores [14], especially when taking into account the transmission characteristics of microscope optics and the wavelength range of detectors. Many modern chemical fluorophores have well-defined excitation spectra and quantum yields in excess of 0.9. Hence, much attention has been focussed on maximising photostability.

Photostability has two components. The first of these, as mentioned above is photobleaching. In this process, fluorophores become structurally altered via a light-induced reaction and hence become non-fluorescent. This can be observed in the temporal fluorescence emission of a single molecule as a stepwise reduction in fluorescence intensity. Whilst initially seeming disadvantageous, the photobleaching effect can be leveraged in order to determine the number of fluorescent proteins within a single diffraction-limited spot (see below). The second component of photostability is photoblinking. In this situation there are intermittent changes in the intensity of the fluorophore that can hamper accurate measurement of single molecules if it occurs on the same timescale as the measurements are being performed. However, these properties can also be exploited, for example in Stochastic Optical Reconstruction Microscopy (STORM) [15] where the stochastic blinking or switching of fluorophores is used to effectively image isolated molecules within an otherwise diffraction limited collection, in order to build up a super-resolved image through sequential spot detection and localisation.

The most obviously final consideration is the excitation and emission wavelength. In addition to the practical considerations with respect to the light sources, filters and detectors available in a particular setup, consideration must be made for bleedthrough as outlined above. Additionally, if FRET experiments are to be performed, the choice of fluorophore pair is important. FRET is the nonradiative transfer of energy from a donor fluorophore to an acceptor fluorophore with an associated decrease in donor emission and concomitant increase in acceptor emission [16, 17], hence fluorophore pairs must be chosen with appropriate spectral overlap. Care must be taken to minimise crosstalk – the direct excitation of the acceptor fluorophore with the wavelength used to excite the donor.

There are now a wide variety of high-quality, commercially-available fluorophores with excellent spectral properties. Therefore, one of the main challenges in single molecule analysis of fluorescent proteins is the labelling of the protein itself. A number of outcomes must be achieved – the protein must still be fully functional when labelled, the labelling site must be available for high efficiency labelling and the protein must only be labelled at a single, often defined, site. A number of biochemical approaches have been developed to allow such labelling, each with advantages and limitations (Table 1).

An obvious first port-of-call when fluorescently labelling proteins is fusion to intrinsically fluorescent proteins e.g. GFP / mCherry and their derivatives [18]. The significant advantage of this approach is that the fluorophores can be incorporated into the protein of interest by translational fusion which can be accomplished *in vivo*. Additionally, these fluorescent proteins are well characterised and derivatives are available that span the visual spectrum [18]. However, these fluorescent proteins are relatively large and have the potential to degrade. Their large size also generally necessitates the use of linkers between the membrane protein and the fluorophore, reducing the resolution of any spatial information depending on the approach used. These fluorophores are generally not particularly bright and, although optimisation has been performed, they may well never match chemical fluorophores in this regard. Finally, the fluorophores can form oligomers [19] which can be problematic when determining protein-protein interactions, especially when these may be relatively weak in membrane proteins.

A number of tags exist that can be inserted into proteins and then their endogenous enzymatic activity used to specifically link a fluorescent substrate to a known location in the protein of interest. For example, SNAP-tags [20, 21] react with benzyl-guanines (BGs) which may be modified by addition of a fluorophore to the benzyl group. Similarly, Halo tags [22] can be labelled with chloro-alkane derivatives. These approaches can be used to label purified proteins or those *in vivo*, with membrane-permeable dyes allowing cytoplasmic labelling. A limitation of this approach is that the tags must be relatively large in order to maintain enzymatic activity and the range of fluorescent substrates is not as wide as for other approaches. Several relatively small tags exist that can be fused to the protein of interest and enzymatically labelled. These tags include peptide carrier domain (conjugates via Sfp phosphopantetheinyl transferase), Q-tag (targeted by transglutaminase) and the biarsenical-

tetracysteine system (CCPGCC) that react with FAsH and ReAsH compounds [23]. The advantage of these compared to SNAP and Halo tags is that the enzymatic activity is exogenous to the protein to be labelled so the tags are smaller. An additional option is direct labelling of hexahistidine tags using  $(\text{Ni}^{2+}:\text{nitrilotriacetic acid})_n$ -fluorochrome conjugates [24] with the advantage that many protein purification strategies already include a site-specific hexahistidine tag. A limitation of many of these tagging options is a relatively limited number of available fluorescent conjugates.

If the protein to be labelled has been purified then it is common to use site-directed labelling of certain amino acids. Lysine is one option where the  $\epsilon$ -amino group can be labelled with amine-reactive conjugates e.g. succinimidyl esters. However, lysine is a relatively high abundance amino acid even within hydrophobic membrane proteins which can result in multiple labelling sites. Perhaps a more favourable choice for direct labelling of amino acids is cysteine [25] whereby the thiol group can be irreversibly conjugated to maleimide-containing fluorophores. Alternatively iodoacetamide conjugates can be used. For both reactions, it is first necessary to reduce the thiol group using a biologically-tolerated reductant e.g. DTT or TCEP. It is also often desirable to remove excess reductant to ensure the reaction proceeds efficiently [26]. To enable single, site-directed labelling of particular residues, it is first necessary to mutagenize endogenous cysteines. Generally, those within the bilayer will not label particularly efficiently and it is often essential to leave those involved in disulphide bond formation as these tend to be important for the structural integrity and hence activity of the protein. Cysteines are normally altered to either alanine or serine and then activity of the cysteine-depleted protein should be assessed e.g. in the case of G protein-coupled receptors the purified, cysteine-depleted variant should be pharmacologically comparable to the wild-type. In order to assess the labelling efficiency and the accessibility of any remaining cysteines, a PEG-maleimide band-shift assay [27] can be employed which enables calculation of both the number of binding sites and the optimal labelling conditions. Efficiency of labelling with small molecule maleimide fluorophores can be calculated by comparison of the  $A_{280}$  and  $A_{\text{ex}}$  (excitation maximum) with appropriate consideration of the contribution of the fluorophore to the  $A_{280}$ .

A further option incorporates unnatural amino acids (UAAs) into target proteins. This emerging technique expands upon the largely conserved paradigm [28] by site-specifically mutating canonical residues [29]. This growing family of chemically or bio-synthetically engineered molecules possess functional groups absent from nature; revealing manipulative applications including PEGylation, photo-crosslinking and FRET studies [30-32]. Following site-directed mutagenesis to incorporate the amber stop codon (TAG), a dedicated set of components are also required in order to incorporate the chosen acid at that position. A mutually orthogonal pair of tRNA and its corresponding aminoacyl-tRNA synthetase [33] are utilised to prohibit crosstalk with endogenous transcription machinery. UAAs therefore offer a specific and highly controllable *in vivo* method of probing protein structure, function and interactions.

UAAs possessing inherent fluorescence may complement existing single molecule imaging and dynamics. Dansylalanine produces increased fluorescence intensity under hydrophobic conditions [34], L-(7-hydroxy-coumarin-4-yl)-ethylglycine is pH sensitive [35] while p-nitrophenylalanine quenches proximate tryptophan residues [36]. These examples are therefore proposed as probes for solvent exposure, helix destabilisation and conformational change, respectively. Subsequently developed were a pair of optimised UAAs, enabling highly specific, spontaneous differential fluorescent labelling and FRET between the two [32]. Utilising FITC and TRITC conjugates, FRET was observed, sensitive to denaturation by increasing urea concentration. To overcome kinetic limitations, spontaneous labelling utilised the principle of the Diels-Alder reaction; a form of cycloaddition possible under reasonable physiological conditions [37, 38]. Furthermore, incorporation of CouA into glutamine-binding protein (GlnBP) was offered as a viable FRET pair with GFP [39]. This provides an alternative to disruptively large fluorophores and terminal fusion proteins which may contribute to steric hindrance. A wider variety of additional UAAs with unique properties is essential for further expanding the study of single molecules; an optimised screening method identified several new possibilities for the incorporation of these probes into *E. coli*-expressed GFP [40].

Depending on the activity of the protein of interest it can also be possible to label the ligand. For example, G protein-coupled receptors have been studied using labelled ligands. Peptide ligands can easily be labelled during synthesis with a number of fluorophores. There are also now an array of commercially-available ligands for a range of receptors. There are considerations when using labelled ligands. The affinity of the ligand must be determined, as must its ability to fully activate the receptor to ensure the relevance of any results. Due to the likelihood of random orientations of the receptor in the DIB, if ligand is added to one side of the bilayer this may only label, statistically, 50% of the receptors. However, ligand could be pre-bound to the receptor ensuring maximal labelling (depending on the fractional occupancy of the receptor). In this case the equilibrium of free and bound ligand must be carefully considered to ensure that receptors stay labelled during the imaging process.

### ***Generation of the bilayers***

The formation of DIBs exploits the spontaneous self-assembly of lipid at a water-oil interface. An aqueous droplet submerged in an oil environment in the presence of lipid will acquire a self-assembled lipid monolayer due to the amphiphilic nature of the lipid. Bringing two such interfacial monolayers into contact forms a bilayer [11]. If solubilised membrane proteins have been incorporated into one of the droplets then they will become incorporated into the bilayer [11]. By this process it is relatively straightforward to create bilayers containing purified membrane proteins and their complexes. DIBs have often been made by contacting two or more aqueous droplets to form bilayers or to make bilayer networks for electrophysiology measurements [11, 41] or assemble soft matter devices [42, 43]. Such constructs can take on interesting properties augmented by biological function of membrane proteins.

However, In order to use DIBs for biophysical imaging, especially at the single molecule level, it is desirable to bring the bilayer into close proximity with a glass coverslip to interface with single molecule microscopy techniques such as total internal reflection fluorescence (TIRF). A route to achieving this is to create a bilayer between an aqueous droplet and hydrogel support submerged in an oil environment (Figure 1a). In this scenario, a spin-coated hydrogel layer, often consisting of agarose, is used as an aqueous support to assemble a monolayer of lipids when submerged in a hydrophobic solvent such as hexadecane. A microfabricated device may be used to contain the oil and allow production of multiple bilayers within the same setup. The addition of a monolayer-coated aqueous droplet into the hydrophobic phase, where the droplet descends under gravity, results in the droplet contacting the monolayer of the hydrogel support and the spontaneous formation of a bilayer (Figure 1b). The incorporation of an aqueous reservoir in the form of bulk hydrogel in contact with the thin supporting agarose layer, provides hydration and a supply of salts or reagents to the underside of the bilayer. This represents a major advance over more traditional supported lipid bilayer approaches, such as those formed by vesicle fusion on glass. A comprehensive review of the methods required for formation of such DIBs can be found in [44]. In these cases access to the underside of the bilayer is not possible due to the direct interaction between lipid and support, precluding trans-membrane protein insertion, free diffusion of membrane components, and electrical and chemical access to the underside of the bilayer. Conversely, the majority of approaches affording these properties are not readily amenable to single molecule imaging (e.g. Montal Muller bilayers). In contrast DIBs retain these properties through the hydrogel support and by spin coating this agarose layer on a coverslip the hydrogel remains sufficiently thin to enable TIRF imaging via an evanescent field propagating ~200 nm above the coverslip surface. This enables dynamic high signal:noise fluorescent measurements at the membrane as illumination is constrained to a narrow plane precisely at the location of the bilayer. In this way single molecule resolution can be achieved. This allows a range of fluorescence techniques to be applied to the membrane, with the ability to exert significant control over membrane components, aqueous environment and physical properties such as the size of the bilayer. Thus in this format DIBs can facilitate a host of emerging opportunities for single molecule measurements of membrane proteins.

Functional membrane proteins have been reconstituted into DIBs in a number of ways. Whilst the mechanism by which proteins are incorporated into the DIB is relatively unknown, there are several mechanisms by which this incorporation can be achieved or enhanced. Detergent solubilised protein may be reconstituted by inclusion in the aqueous droplet prior to bilayer formation. Similarly, reconstitution directly from unpurified membrane fragments may be achieved by deposition within the underlying agarose prior to bilayer formation [45], or via inclusion in the aqueous droplet. In a not-dissimilar approach, successful protein reconstitution has been demonstrated directly from electrophoretic gels following DIB formation directly on the gel surface. In Vitro Transcription & Translation (IVTT) has been used for *in situ* protein synthesis and spontaneous incorporation in droplet-droplet DIBs, for relatively simple membrane proteins that fold spontaneously, such as KcsA and alpha hemolysin [11]. Similarly, protein may be reconstitution from proteoliposomes through their incorporation in the aqueous droplet [46]. Single molecule fluorescent imaging can be used to confirm membrane protein incorporation with the observation of 2D diffusion of membrane associated species. Electrophysiology or ligand binding may be used to demonstrate functionality. As in other membrane systems the expression and purification of membrane proteins can represent a major challenge ahead of reconstitution, however from this point several variables can be explored to optimise reconstitution in DIBs. In the case of purified protein, detergent concentration is clearly a significant consideration. Detergent or other amphiphiles are usually required to stabilise the membrane protein structure. However, such materials may also be destabilising to lipid bilayers. Dilution of protein into the droplet mixture immediately prior to droplet incubation and monolayer assembly, can serve to dilute the detergent (below the cmc) which appears to enhance membrane protein insertion. We have hypothesised that the choice of detergent and its off-rate can help ensure sufficient protein stability for the short period of droplet incubation and bilayer formation, whilst limiting total detergent in the system. It should be noted that in an oil-water system in the presence of excess lipid, the cmc and aqueous concentration of detergent will likely be difficult to predict from simple aqueous only data that is common in the literature. Droplet incubation time, lipid concentration and oil phase properties can all impact on time for lipid monolayer assembly and resulting bilayer stability, tuning of these variables in conjunction with protein and detergent concentrations can be used to maximise protein insertion and bilayer stability.

Two methods exist for the creation of DIBs; termed lipid-out in which lipids are dissolved in the oil, or lipid-in in which lipid vesicles are present in the aqueous droplet along with the protein of interest. This affords opportunity to address each lipid monolayer compositions individually, enabling the creation of asymmetric bilayers [47]. Similarly, the contents of each aqueous phase, whether droplet or hydrogel, can be controlled without mixing between the compartments. A significant advantage of DIBs can be their stability compared to other forms of artificial bilayers. They have been demonstrated to be stable for days to weeks which enables repeated analysis and also removes any restrictions when imaging membrane protein systems on the biological timescale. Droplets can also be moved using an incorporated probe or electrode. Perhaps the most useful application of this in terms of studying membrane proteins is to allow control over the bilayer area and hence protein density [48, 49]. This allows the effect of membrane protein concentration to be investigated within a single sample. The ability to create DIBs up to 1 cm in diameter, allows a large number of protein molecules to be incorporated and analysed without generating excessive protein densities. DIB membranes are able to form gigaohm seals, making them amenable to single channel electrophysiology measurements or voltage application whilst providing assurance of segregation of contents either side of the bilayer. The ability to insert electrodes or probes into the droplet enables nano-injection of reagents [11], opening the opportunity to make measurements on a single protein population following dynamic changes to the local chemical environment. It should also be possible to extend this principle to complete droplet perfusion.

It is becoming increasingly apparent that a significant proportion of membrane proteins are affected by the composition of the surrounding lipid bilayer. This may be due to specific lipid-protein contacts but also more global effects such as membrane thickness and fluidity [39]. Lipid composition is therefore a vital consideration when generating the bilayers. Endogenous lipid bilayers are heterologous mixtures of large numbers of different lipid species and can also be asymmetric with

respect to the lipid composition of each leaflet. Theoretically, it is possible to recreate such complexity within DIBs. Whilst there are some practical limitations (outlined below), bilayers have been reported from a number of different lipids and lipid mixtures [44], including phase separated systems [50]. Dynamic control of lipid composition by titration of dissolved lipid in the oil phase has been demonstrated [51], which should be applicable to dynamic membrane protein measurements.

The combination of the above control of the *in vitro* membrane environment together with single molecule imaging can be used to quantify dynamic changes on a single protein population. Multicolour imaging can correlate two or more species or processes simultaneously. Furthermore simultaneous and correlated electrophysiology and optical measurements can be made [52] which have been proposed could be used in unison to give complementary structural and functional information [51]. Single molecule FRET can be used to quantify protein-protein interactions and ion-flux imaging in DIBs (optical patch-clamping) has been demonstrated to give comparable resolution to electrical measurements [53]. A number of fundamental single molecule approaches and concepts underpin these experimental capabilities, and these are introduced below. Further, since DIBs in this format interface with standard microscopy coverslips, many other emerging microscopy and optical approaches can be applied to membrane protein study in DIBs.

### ***Methodologies***

Single molecule imaging approaches have been reviewed elsewhere and their complete coverage is beyond the scope of this review. A brief introduction to some key methodologies applicable to membrane protein imaging in DIBs is outlined in the following section.

#### **Single particle tracking**

A combination of DIBs and total internal reflection fluorescence (TIRF) microscopy can be used to track single membrane protein particles. In contrast to epifluorescence in which the laser is directed at 90° to the coverslip, in TIRF the laser is angled such that it illuminates only ~200 nm depth of the sample. This effectively means that only those proteins incorporated into the DIB are imaged. By employing a high-sensitivity emCCD camera, single fluorophores can be imaged with temporal resolution on the order of 2-30 ms. The fluorophore chosen for such studies must be highly resistant to photobleaching to ensure that single particles can be tracked throughout the desired timeframe.

Once imaged, image analysis is necessary to detect single molecule spots and extract quantitative information on their intensity and position. Researchers frequently use custom-developed code to achieve this, although increasingly comprehensive open source packages are available in the versatile imageJ [54] build FIJI [55]. Challenges in accurate single molecule spot detection include the identification of often low signal:noise diffraction limited spots on a noisy background. Spot identification may use spatial filtering techniques to enhance spot detection, prior to 2D Gaussian fitting to extract sub-diffraction limit spatial location and fluorescence intensity. From this data, energy minimised temporal linking of spots in successive frames may be employed to track individual molecules, their diffusion and colocalisation. Such algorithms often need to be optimised for the experiment in hand, with ability to accommodate blinking (missing frames) and specify limits on maximum likelihood of diffusional distances between frames. From these trajectories diffusion coefficients can be extracted from the mean squared displacement (MSD) plots. We have been able to use such approaches to differentiate between monomeric and dimeric protein populations on the basis of diffusion coefficients that scale with molecular radius. Spatial position may be used in conjunction with intensity data to provide further insight and spatially registered multi-coloured imaging may also be used to characterise complementary processes or molecular interactions. Given the diffraction limited nature of single molecule imaging, colocalisation of multiple species becomes a challenge to characterise beyond ~200 nm but the use of single molecule FRET as discussed below can be used to improve confidence of interaction (~5 nm) and quantitative FRET measurements may give insight into conformational dynamics (Figure 1c) [56].

#### **Step-wise photobleaching**



Due to the diffraction limit, it is impossible to resolve particles which are within very close proximity. A key biological question regarding membrane proteins is often their ability, or requirement, to form complexes. Whether these are homo- or hetero-oligomeric, it is possible to resolve the stoichiometry via step-wise photobleaching. Whilst FRET (see below) is capable of providing some information regarding protein stoichiometry, it requires differential labelling of the samples and downstream analysis of the data and is generally limited to two protein partners. FRET is also often better suited to analysing protein kinetics and configuration. The simplicity of step-wise photobleaching is highly attractive for these types of studies.

When observing single particles, photobleaching is evidenced as a step-wise reduction in fluorescence intensity [57]. Assuming that each subunit in a complex is labelled with a single fluorophore, the number of photobleaching steps that occur for a particular particle will be equal to the number of individual proteins in the complex. However, in reality not all photobleaching steps will be seen during the experimental timeframe and not all subunits in the complex will be labelled. Assuming that no subunit is labelled more than once, the maximal number of observed bleaching steps can be no greater than the number of proteins in the complex. Statistical analysis of a binomial distribution can then be used to determine the actual number of proteins in the complex. This is a quick and effective analysis method for oligomeric complexes [58, 59].

### **FRET**

FRET can provide further insights into the formation of membrane proteins complexes. FRET analysis of protein complexes requires incorporation of differentially-labelled donor and acceptor subunits within the same DIB. Fluorescence of the acceptor-labelled molecule is only observed if the excited donor-labelled molecule is in close proximity (~3-12 nm for typical FRET pairs). FRET thus presents an advantage in reliably determining protein-protein interactions over commonly used diffraction-limited co-localisation techniques which may interpret protomer crowding as oligomerisation. While super-resolution imaging can significantly improve spatial resolution (< 10 nm) of co-localisation analyses, e.g. as employed for the study of GPCR dimerisation [60], it comes at a cost of temporal resolution, thus hampering kinetic studies of complex formation. Indeed, the combination of high spatial and temporal resolution of single-molecule FRET imaging makes it a powerful technique to study the dynamics of protein complex formation [61], allowing e.g. the determination of on-rates, dissociation constants, and the lifetimes of transient protein complexes. Furthermore, by measuring both donor and acceptor fluorescence, the proportion of monomeric and oligomeric species can be probed. FRET in combination with reconstitution in DIBs can thus be used to study the regulation of complex formation, for example as a function of protein density, lipid composition of the DIBs or the presence of modulators. It should be noted that there are particular challenges and consideration when analysing single molecule FRET data. Whilst this is beyond the scope of this manuscript, other excellent reviews are available e.g. [62]

Because FRET efficiency is proportional to label separation, FRET analysis using site-direct labelling with small fluorescent probes can be used for structural analysis of protein complexes, for example to map out the interaction interface by placing probes on different sites. Furthermore, it may give insights into complex heterogeneity, as complexes with different inter-label distances give rise to differences in acceptor emission intensity which can be detected. In addition to complex formation, FRET may be used to study conformational changes within stable (low  $K_d$ ) complexes, by observing changes in FRET efficiency e.g. in the presence or absence of agonist. Intramolecular conformational changes of membrane proteins may also be assessed, if a differential site-specific labelling can be achieved.

### ***Limitations***

A potentially significant limitation of the reconstituted DIB system is that shared by any artificial membrane system, in that only the components that have been actively reconstituted are present. The living cell is a highly complex environment and any artificial system, by its very nature, will lose some of this complexity. This has both advantages and disadvantages to studying membrane proteins. Disadvantages include the fact that some key, possibly unknown, component will be missing that

regulate protein activity or dynamics *in vivo*. However, this can also be an advantage as studying simplified systems presents an opportunity to investigate the interaction of individual components. For example, dynamics of interactions can be studied or the effects of different lipid compositions on protein activity.

There is also an unresolved question regarding the insertional direction of the proteins of interest. In some cases this will not matter, for example if truly monomeric proteins are being studied in a symmetric bilayer there should be no effects from this asymmetric protein insertion. However, in cases where protein-protein interactions are studied, or the membrane is asymmetric, this effect will need to be considered. Although interactions between oppositely-oriented membrane proteins should never occur *in vivo*, it may be possible to detect spurious interactions in this system.

### ***Hints for troubleshooting***

Membrane protein expression and purification is notoriously difficult. However, there are a variety of systems available which include *E. coli*, mammalian cells, baculovirus/insect cells, yeast and cell-free expression systems. Each of these have advantages and disadvantages for expression of membrane proteins and have been reviewed elsewhere [63]. However, as a guide, it is better to produce eukaryotic proteins in eukaryotic systems and likewise for prokaryotic proteins. Due to the complexity of membrane proteins, the importance of post-translational modifications and the lipid environment for correct folding and activity, the host cell should be carefully chosen. In a similar manner, it is important to carefully consider the purification strategy for membrane proteins. An affinity tag is often used but the site of this can influence protein activity and hence should be sited based on available biological data. Likewise, the purification strategy should be optimised. The use of detergents is commonplace [64] but can lead to reduction of protein activity through loss of the lipids and associated lateral pressure. A more recent development in the purification of membrane proteins is the use of styrene maleic acid lipid particles (SMALPs) which retain the bilayer [65]. However, there are no reports of transfer of a protein from a SMALP to a DIB so there are caveats with this approach in that it may ultimately be necessary to add detergent to enable effective reconstitution in the model bilayers.

The labelling strategy for membrane proteins must be carefully considered. As with purification tags, if tags are to be used for labelling they should be placed where they will be exposed sufficiently to allow efficient labelling but also should not interfere with the native activity of the protein. If site-directed labelling is to be employed then there are a number of key considerations [25]. Firstly, there should be no endogenous sites that would be labelled as this would result in multiple labels per protein which is undesirable for single molecule approaches including photobleaching and FRET. To enable analysis of the stoichiometry of labelling, fluorescence assays can give a population-level indication but PEG-maleimide labels can be used, along with gel bandshift assays [27], to determine the stoichiometry of individual molecules. The caveat is that PEG-maleimide is relatively large compared to small molecule fluorophores and hence may not accurately reflect labelling accessibility and kinetics. Secondly, it is important that the chosen site is accessible to the fluorophores for labelling and, at least in the case of FRET, relatively rigid within the protein structure e.g. not within a loop. As such, the end of transmembrane helices, which can be predicted from hydrophobicity analysis and comparison to existing crystal structures, can provide ideal sites. It should be noted that whilst chemical fluorophores are relatively small, they are still substantially larger than amino acid side chains and are thus capable of significantly impacting on protein structure if poorly located. When using unusual amino acids there are a number of controls which must be performed including the production of analogous mutants. These must possess a residue structurally similar to the unnatural acid in question but lacking the functional group, along with comparison to wild type. Important considerations include polarity, cyclic structures, hydrophobicity and spatial orientation; for example, lipid-facing FRET pairs may help to preserve signal sensitivity.

If modifications have been made to the protein to enable fluorescent labelling then it is vital to ensure that the protein is still functional in its final form. Ideally this will be done for the final, labelled

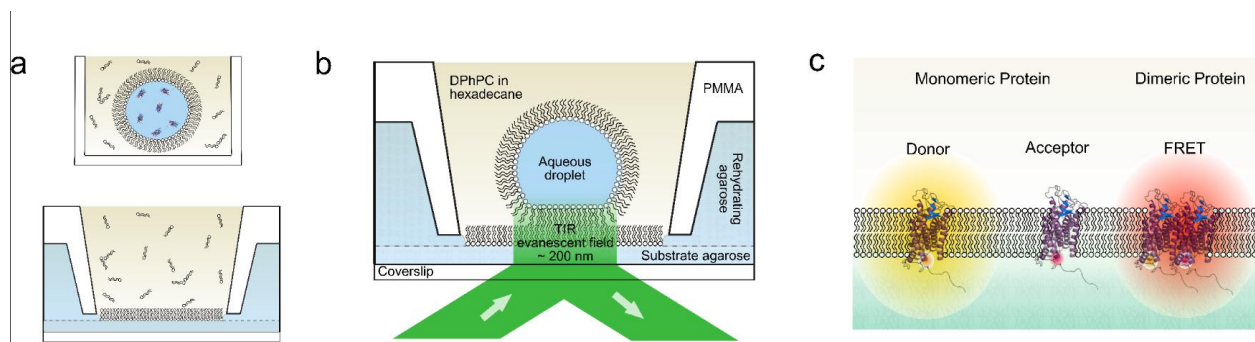
protein. For some classes of protein this can be relatively straightforward e.g. G protein-coupled receptors can be assayed for both ligand binding and G protein activation *in vitro*. It is also possible to test the transport capabilities of a range of transport proteins using liposomes or similar assays. However, other classes of proteins may be more difficult to directly assay, especially if they are dependent on protein-protein interactions with partners lacking from the purified system or have other functional roles. If activity of the labelled protein cannot be robustly demonstrated in the labelled form then results of single molecule studies will need to be interpreted with caution.

It is also important to consider the density of the protein in the DIB. If proteins are too dense then it is possible that non-specific interactions will be observed rather than those which represent physiologically-relevant events. Additionally, the protein-lipid ratio can be vital to ensure that there is sufficient bilayer to allow diffusion of the membrane proteins and provide sufficient annular lipids such that proteins are not artificially corralled through a lack of solvent. In the case of homo-oligomeric complex formation, an important practical consideration is the ratio of donor to acceptor molecules. By reconstituting acceptor-labelled protomers in excess of donor-labelled protomers, the proportion of FRET-capable (donor-acceptor) oligomers relative to donor-donor oligomers can be increased. Additionally, the reconstitution efficiency and resulting protein densities need to be considered; if the dissociation constant of oligomerisation is high, complex formation may be too rare to observe at low protein density.

Traditionally, DIBs have been formed from DPhPC which is naturally found in archaea [66] and therefore could be assumed to have limited relevance for study of bacterial and eukaryotic proteins. If DPhPC is to be used, the functional activity of the protein of interest should be determined in this lipid environment. For example, the ability of a receptor to bind ligand and to functionally activate downstream signalling components should be assessed. We have used a variety of lipid compositions within DIBs and whilst many form stable bilayers, occasionally “blebbing” is observed and the bilayers become unstable. To date, there is no comprehensive analysis of lipid compositions that can be used to form stable DIBs and therefore the approach taken should use any prior knowledge of the lipid requirements for a particular protein which may be determined from other experimental systems such as liposomes and planar bilayers.

**Table 1:** Single molecule labelling strategies for membrane proteins.

Technique	Advantages	Disadvantages	Examples
Fusion proteins	Incorporation as translational fusion.  Well characterised proteins.  Span the visual spectrum.	Relatively large.  May degrade.  Required linker sequences.  Not very bright.  May oligomerise.	GFP and derivatives.  mCherry and derivatives.
Protein / peptide tag	Specific modification.  <i>In vivo</i> purification.  Specific labelling sites.	Relatively large.  Narrow range of fluorophores.	SNAP tags  Halo tags.  Q-tag.  Biarsenical-tetracysteine system
Site-directed labelling	Extremely specific locations.  Range of high-quality fluorophores available.  Small modification.	Amino acids to be labelled may be relatively high abundance.  Amino-acid-depleted protein variants may have altered activity.	Lysine and cysteine-reactive dyes.
Unnatural amino acids	Extremely specific locations.  Small modification.	High-resolution structural information is desirable.  Limited range of fluorophores.	Variety of fluorophore conjugates available.
Ligand labelling	Highly specific.  May be commercially-available.	May alter ligand properties e.g. affinity.  Only suitable for selected membrane proteins.	Peptide-based GPCR ligands.



**Figure 1: Single Molecule Imaging in droplet interface bilayers (DIBs):** a) An agarose coated coverslip (bottom) and an aqueous droplet containing detergent stabilised protein (top) are separately incubated in lipid in alkane solution. A lipid monolayer spontaneously self-assembles at each water-oil interface. The aqueous droplet is subsequently transferred into the well of the PMMA device housing the agarose-coated coverslip substrate. b) A lipid bilayer forms on the contacting of the two self-assembled monolayers of the droplet and agarose layer. Purified membrane proteins present in the droplet integrate into the resultant bilayer. Total internal reflection of laser light at the glass-sample interface generates an evanescent field illuminating the membrane enabling fluorescent imaging. c) Total internal reflection fluorescence (TIRF) enables single molecule imaging of membrane proteins reconstituted into DIBs. This schematic illustrates the use of single molecule FRET to image donor-labeled monomeric protein and FRET-capable donor and acceptor labeled dimeric protein complexes at the single molecule level. An approach adopted to elucidate the dimerisation dynamics of the GPCR, NTS1, in DIBs [56].

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- Fluorescent membrane proteins can be generated through a variety of labelling strategies.
- Droplet hydrogel bilayers allow analysis generation of defined planar bilayers.
- TIRF imaging and biophysical techniques allow study of protein dynamics and interactions.

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