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Advances in the application of fluorescence correlation spectroscopy to study detergent purified and encapsulated membrane proteins

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ABSTRACT

Fluorescence correlation spectroscopy (FCS) is a quantitative spectroscopy technique which could potentially increase throughput and sensitivity of screening for ligand, substrate and inhibitor binding to membrane proteins in solution. However, the purification of membrane proteins in their active forms is complex, as the lipid bilayer provides stability and its removal often causes the protein to become conformationally unstable. This has limited the application of biophysical techniques such as FCS to study the function of membrane proteins. The recent application of native extraction techniques such as styrene maleic acid lipid particles (SMALPs) has resolved this issue and FCS has emerged as a powerful option for studying proteins extracted in this way. This review will discuss the application of FCS to study purified membrane proteins in detergent micelles, nanodiscs and SMALPs and its potential to be used routinely in membrane protein drug discovery.

1. Introduction

Membrane proteins, such as receptors and transporters, are important drug targets and a major focus for new drug development. As such, it is important to develop new ways to analyse their interactions with drugs, in particular for screening purposes. Whilst there has been an emphasis on developing new methods to determine their pharmacology in live cells (Stoddart et al., 2015; Sykes and Charlton, 2018), the use of purified proteins allows characterisation of drug-target interactions in a defined, isolated environment. This allows fine control over experimental conditions, such as the concentrations of substrate, drugs and co-factors, and the ionic environment, as well as allowing access to both extra- and intra-cellular faces of the target. Sensitive biophysical techniques such as surface plasmon resonance (SPR) and fluorescence correlation spectroscopy (FCS) have been successfully used for both soluble cytosolic proteins and integral membrane proteins (Patching, 2014; Machán and Wohland, 2014). However, the application of such approaches to study membrane proteins has been limited by their inherent instability when removed from their lipid environment. More recently, the use of thermo-stablising mutations and, in particular, extraction and encapsulation techniques which allow the inclusion of lipids, such as nanodiscs and styrene maleic acid lipid particles (SMALPs), has removed some of these barriers (Antoine et al., 2016; Denisov et al., 2004; Knowles et al., 2009). This review focusses specifically on recent developments in the use of FCS to study purified membrane proteins and its potential future use in characterisation of their pharmacology and function.

2. Fluorescence correlation spectroscopy

FCS is a quantitative spectroscopy technique which enables the concentration and diffusion properties of fluorescently labelled species to be determined with single molecule sensitivity (Magde et al., 1972; Berland, 1995). Using a standard confocal optical set-up, a laser is focussed to a diffraction limited spot through a high numerical aperture objective lens, with emitted light collected through a confocal pinhole, defining a small observation volume of approximately 0.2 fL (Fig. 1(a)). Photons are recorded in a time-dependent manner as they are emitted

Abbreviations: FCS, Fluorescence Correlation Spectroscopy; FCCS, Fluorescence Cross Correlation Spectroscopy; GPCR, G Protein-Coupled Receptor; GPMV, Giant Plasma Membrane Vesicle; GUV, Giant Unilamellar Vesicle; PCH, Photon Counting Histogram; SMALP, Stryene Maleic Acid Lipid Particle; VLiPs, Virus-Like Particles.

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from fluorescent species that diffuse through the observation volume, creating fluorescence fluctuations. These fluctuations can be analysed in two ways. Analysis of their time-dependency, using autocorrelation analysis, yields information about the diffusion speed and concentration of the fluorescent species, whilst analysis of their amplitude by photon counting histogram analysis (PCH) allows its concentration and molecular brightness to be quantified (see Fig. 1(a) and its legend for further explanation and visualisation), (Huang et al., 2004). These

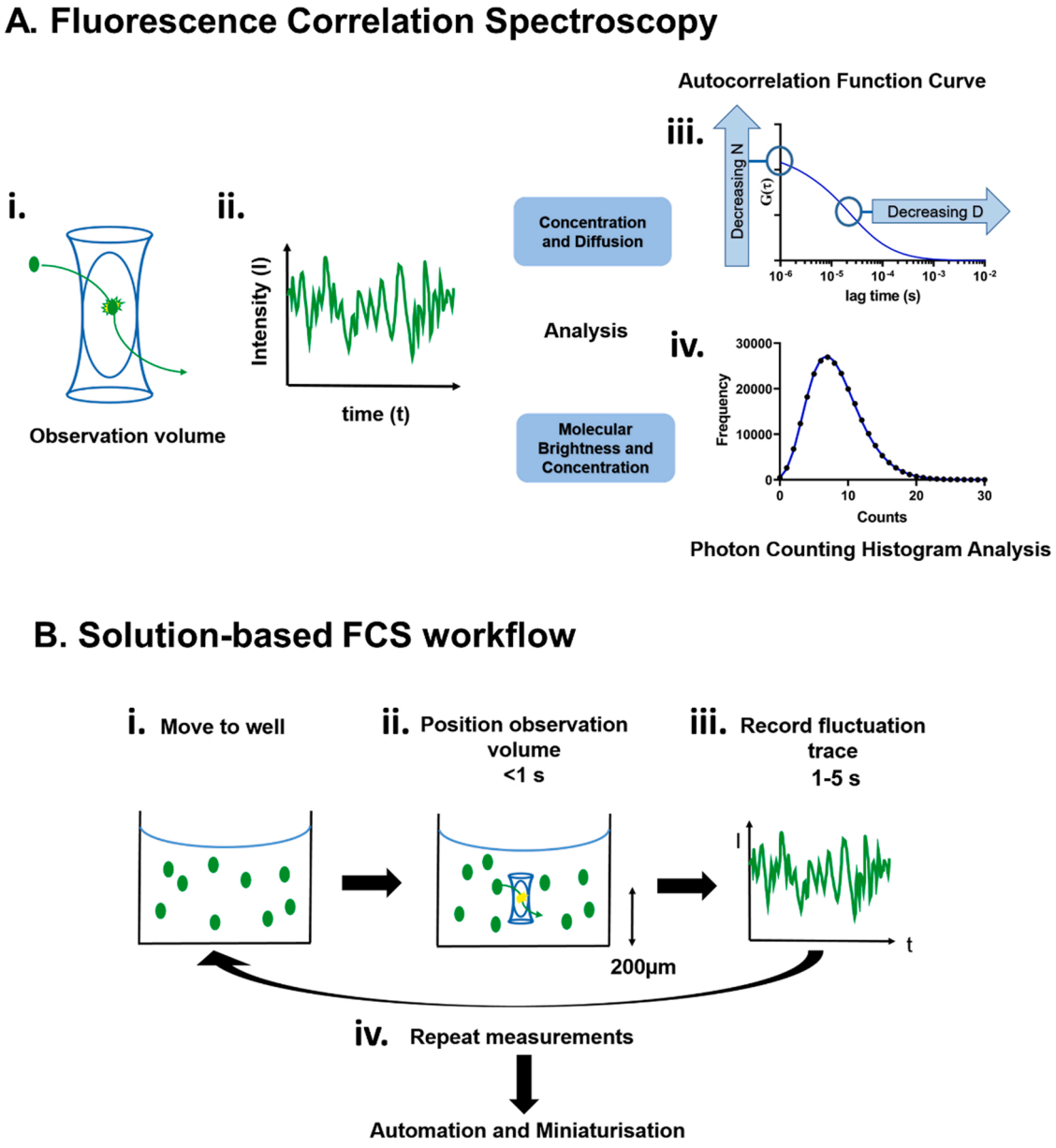

Fig. 1. Fluorescence Correlation Spectroscopy. (A) Fluorescence correlation spectroscopy (FCS) measures the fluctuations in fluorescence as a fluorescent species (green oval) passes through a confocally defined observation volume of approximately 0.2 fL. (i) Within the observation volume the fluorescent species is excited by incident laser excitation and emits photons which are recorded by sensitive photon-counting detectors. (ii) Fluorescence fluctuation traces are recorded detailing intensity (I) counts over time (t). Small species will move faster through the observation volume and will have a higher frequency of fluctuations. Species with multiple fluorophores will have greater amplitude of intensity fluctuations. (iii) Fluctuation traces can be analysed by construction of an autocorrelation curve from which the average diffusion and concentration can be calculated. The half-decay of the curve provides the dwell time of the species, with slower species shifting the curve rightwards. The y-intercept gives the inverse of the average number of particles (N) within the observation volume; the curve amplitude increases as the particle number decreases. Both can be quantified to provide the diffusion coefficient (D) and concentration following calibration of the detection volume with a dye of known diffusion properties. (iv) Fluctuations can also be analysed by Photon Counting Histogram analysis in which the super-Poissonian behaviour of the constructed histogram of fluorescent fluctuations can be modelled to determine average molecular brightness and concentration. (B) Solution-based FCS workflow. i) The chamber or well containing solution of fluorescent species is positioned on the microscope set-up. Here we represent a 0.7 cm^2 well which will comfortably hold 200 µL. ii) The observation volume is placed ~200 µm above the well bottom to limit reflected and diffracted light from the coverslip. A central position avoids aberrations due to proximity to well edges. If this position has been determined previously, this step can take < 1 s iii) Fluorescent fluctuations, intensity (I) over time (t), are recorded. Trace reads can be short, 1–5 s, membrane proteins diffuse 20–100-fold faster (2–500 µs observation volume dwell time) in solution compared to in live membrane reads. Depending on experiment, solution-based trace reads can be as short as 1 s to allow kinetics studies. iv) Multiple reads can be taken sequentially without significant photo-bleaching due to rapid sample exchange within the observation volume. Solution-based FCS is ideal for miniaturisation and automation, key for higher throughput workflows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complementary analyses with differing sensitivities allow, for example, oligomer stoichiometry to be monitored by changes in molecular brightness which might not be resolved by differences in diffusion co-efficient alone. FCS has both high spatial and temporal resolution, making it ideal for use in small volumes, at low concentrations and applicable to processes over a wide range of time scales. These range from photo-physical changes and chemical reactions (ns-μs) to diffusion (μs-ms), covering diffusion co-efficients from small molecules (200–500 $\mu m^2/s$) and purified proteins (50–100 $\mu m^2/s$) to protein diffusion in cells (1–10 $\mu m^2/s$). However, FCS is not without its limitations and should be seen as complementary to imaging techniques aimed at studying diffusion parameters. Image based fluctuation techniques, e.g. image correlation spectroscopy, cover wider fields of view and are more accurate for slower moving species, whilst single particle tracking is able to monitor diffusion mode and speed of individual particles (Macháň and Wohland, 2014). Super resolution techniques can deliver superior spatial resolution, but do not have the same temporal dynamic range.

FCS was first developed as a biophysical tool to examine the kinetics of ethidium bromide binding to DNA in solution (Magde et al., 1972). However, it was not until 30 years later that instrumentation had progressed sufficiently to not only detect single fluorescent species but also to capture data fast enough to allow its use within biological systems (Berland et al., 1995). These initial steps opened the application of FCS to study protein dynamics in living systems and it has since been widely employed to investigate single molecule interactions and elucidate protein function and organisation (Macháň and Wohland, 2014). In 1999, FCS on the cell membrane was first used to measure the concentration and diffusion properties of both a fluorescent lipid (DiI-$C1_{12}$) and eGFP in the plasma membrane of both plant and mammalian cells, demonstrating that there was sufficient signal to noise above any auto-fluorescence, and photo-bleaching was manageable (Schwille et al., 1999). Whilst most FCS studies on membrane proteins have focussed on the lateral membrane diffusion of the protein itself, it has also been used to determine the stoichiometry of complex membrane proteins and for investigating ligand, substrate and inhibitor binding (Gondin et al., 2019; Herrick-Davis et al., 2013; Corriden et al., 2014). However, several factors render these studies low throughput, including effective protein labelling strategies, careful titration of expression level, membrane placement of the observation volume and photo-toxicity. These factors are generally not limiting for solution-based FCS measurements, which have the added benefit of shorter acquisition reads (Fig. 1(b)).

3. FCS using purified membrane proteins

The purification of membrane proteins in their native state is difficult; the lipid bilayer in which they reside provides stability and modulates their function, and its removal can cause the protein to have limited conformational sampling, mis-fold or even denature (Tehan and Christopher, 2016; Chun et al., 2012). For instance, differences in purification methods for G protein-coupled receptors (GPCRs) have been shown to change the way they interact with ligands, and the pharmacology of many receptors can be changed by lipid components such as cholesterol (Grime et al., 2021; Harwood et al., 2021). This has limited the application of many biophysical techniques, including FCS, to study their function. Notwithstanding this, a small number of studies have used membrane proteins purified in a variety of ways in combination with FCS. The power of FCS to study ligand binding to a membrane protein was demonstrated by an early study on the 5-hydroxytrytamine type 3 A receptor ($5HT_{3A}$), a ligand-gated ion channel (Wohland et al., 1999). Here, the receptor was removed from the membrane using detergents and then affinity purified prior to FCS studies. Using a fluorescent antagonist, the authors showed that only one molecule of the fluorescent ligand was bound per homo-pentameric receptor. FCS has also been used to study the binding of the bacterial surface protein $InIB_{321}$ to a purified single-transmembrane spanning receptor tyrosine kinase, MET (Dietz et al., 2019). Recently, detergent solubilised and purified GPCRs have been used in fluorescence cross-correlation spectroscopy (FCCS) studies (Rico et al., 2019; Antoine et al., 2016). FCCS simultaneously monitors the fluorescence fluctuations of two spectrally separated fluorophores in overlapping observation volumes. By cross-correlating the two fluctuation traces, FCCS can determine if the two species are co-diffusing and hence, interacting (Fig. 2(d)). More specifically, the concentrations of both the free and the interacting species can be simultaneously quantified. Whilst powerful, FCCS is technically more challenging than FCS and requires the careful choice of spectrally separated fluorophores as well as precise overlap of the two detection volumes (discussed further in Macháň and Wohland, 2014). For GPCRs, FCCS has been used to monitor ligand binding to four purified GPCRs; neurotensin receptor type 1, β_2-adrenergic receptor, C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) (Rico et al., 2019; Antoine et al., 2016). In the case of CCR5, FCCS allowed determination of the different binding modes of endogenous and synthetic ligands (Rico et al., 2019). These studies demonstrate the usefulness of solution FCS or FCCS studies to investigate solubilised and purified receptors from the membrane. However, each membrane protein required a different purification method, and some receptors required the introduction of stabilising mutations for sufficient protein yields (Antoine et al., 2016; Rico et al., 2019).

4. Use of membrane patches, vesicles, and nanodiscs in FCS studies

There are a number of alternative methods which can preserve the stabilising lipids surrounding the target protein that are otherwise removed by detergents during membrane protein purification. Swift et al. (2007, 2009), have described a method for preparing membrane patches containing a GPCR, the μ opioid receptor (MOR). They expressed the receptor in Sf9 insect cells using a recombinant baculovirus and, after preparation of crude membrane samples using sonication, used a combination of narrow-gauge syringes and filters to prepare small patches of membranes, termed nano-patches (~100 nm). In a proof-of-principle study, fluorescent and quantum dot labelled MOR ligands were used with FCCS to measure the affinity of these ligands and receptor density in the membrane nano-patches. The nano-patches were found to be of a non-uniform size and contained a much lower number of receptors than expected. This meant an increased number of FCCS reads were required to generate accurate data and the scope for determining further pharmacology was limited (Swift et al., 2007). Alternatively a co-expression system that produces enriched membrane protein samples, termed Virus-Like Particles (VLiPs) has been used with FCS as a model to screen for endothelin A receptor (ET_AR) ligands (Zemanová et al., 2004). Accumulation of the retroviral budding protein, Gag, with the receptor of interest within this insect-cell based co-expression system results in budding of the plasma membrane and production of VLiPs. The VLiPs were relatively large, with a diffusion coefficient of ~3 $\mu m^2/s$, and therefore required long acquisition reads. To combat this, Zemanová et al. (2004) used scanning FCS, where the observation volume is scanned in a line or circle through their sample, and in effect searched for VLiPs and successfully reduced the acquisition read time. However, the VLiPs, like nano-patches, were derived from insect cells, and as such the composition of signalling proteins (Knight and Grigliatti, 2004) and membrane composition (Gimpl et al., 1995) is very different to that found in mammalian cells which can alter receptor function. Using a similar approach to Swift et al. (2007, 2009), but in mammalian cells, Worch et al. (2017), produced nano-patches containing the single-pass transmembrane protein, interleukin-4 receptor alpha chain (IL4Rα) from HEK293 cells. With the aim of reducing the chemical complexity of the system, giant unilamellar vesicles (GUVs) were derived from these nano-patches and used in FCS and FCCS studies to measure the diffusion and binding parameters of a fluorescent ligand to the IL4Rα. These parameters were compared to those using giant plasma membrane vesicles

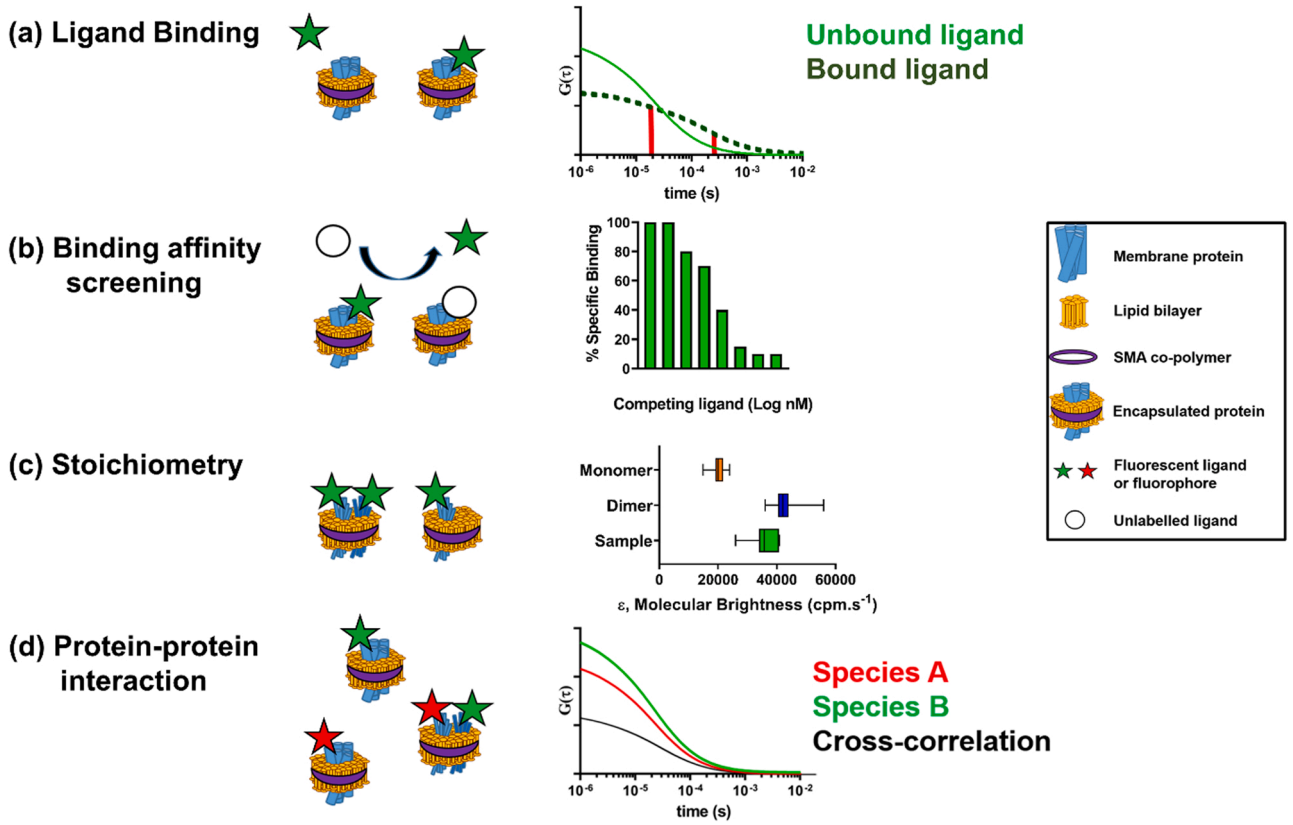

Fig. 2. Applications of SMALPs in solution-based FCS. (a) Ligand binding assay. The diffusion properties of the free fluorescently labelled ligand are determined (green line). In the presence of unlabelled encapsulated protein, a multicomponent diffusion profile is derived (dashed green line) defined by two separate dwell times (red lines). The diffusion and concentration of ligand-bound encapsulated protein can be determined. (b) Competition binding affinity screening. Increasing concentrations of an unlabelled ligand can displace fluorescently labelled tracer ligand from the encapsulated protein. Pharmacologically relevant parameters such as dissociation binding constants (K_i) can be calculated from these data sets. (c) Stoichiometry. The average molecular brightness of a species can be determined by Photon Counting Histogram analysis. Comparison with known controls i.e., monomer (orange) or dimer (blue) can define the stoichiometry of the sample (green). (d) Protein-protein interaction. Fluorescence cross-correlation spectroscopy (FCCS) can determine whether two fluorescently labelled species (fluorescent ligand and/or fluorophore) are diffusing separately or together (black curve). Diffusion coefficients and concentration of all the species can be calculated from the autocorrelation curves. In this example two separately fluorescently-tagged encapsulated receptors are depicted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(GPMVs) and at the plasma membrane of live cells (Worch et al., 2017). GUVs have also been used to monitor the effect of lipid composition on membrane protein diffusion, but in these studies the GUVs were derived from a phospholipid mix and the membrane protein was reconstituted in these GUVs of known lipid content (Ramadurai et al., 2009, 2010). Both GUVs and GPMVs are convenient model membrane systems to study transmembrane proteins using FCS, but due to their large size (10–50 μm), there is still the requirement to manually place the confocal volume at the membrane.

Instead of using the natural lipid components from the cell, stabilising lipids can be added following purification of the membrane protein and, in combination with a membrane scaffold protein, nanodiscs can be formed (Denisov et al., 2004). Nanodiscs containing the ATP binding cassette transporter P-glycoprotein (ABCB1) have been successfully used in FCS studies to monitor the binding of fluorescent substrates for this transporter under different nucleotide bound states (Li et al., 2017). Compared to nano-patches, nanodiscs are uniform in size and homogenous with respect to their lipid composition. Using size exclusion chromatography, it was possible to isolate nanodiscs that contained the target receptor, again ensuring a homogenous solution and reducing the background noise in FCS experiments (Li et al., 2017). However, as a detergent purification step is required prior to nanodisc formation many of the same issues described for purifying membrane proteins are encountered, such as the requirement to introduce thermostabilising mutations and bespoke purification protocols. Despite the limitations in terms of large or non-uniform size, the methodology to produce membrane patches, vesicles and nanodiscs can provide an achievable strategy to preserve the surrounding lipid environment within which to study membrane proteins.

5. FCS studies using SMALP encapsulated membrane proteins

The discovery that the polymer styrene maleic acid (SMA) can be used to directly extract proteins from membranes into SMA lipid particles (SMALPs) (Knowles et al., 2009) has made the application of many biophysical techniques to study membrane protein structure and function much more straightforward (Hothersall et al., 2020). SMALPs are formed by the integration of the hydrophobic SMA polymer into the lipid bilayer leading to extrusion of stabilised discs of membranes of between 10 and 15 nm in size which may contain any integral membrane proteins (Xue et al., 2018). This maintains the surrounding lipid environment and potentially other associated constituent membrane proteins and/or binding partners (depending on size) and allows the study of the target membrane protein in a more physiologically relevant membrane environment. The rapid uptake in the use of SMALPs is in part attributed to the ease of use of SMA; the production of SMALPs from any given cellular background requires little optimisation compared to the reference protocol (Lee et al., 2016; Hothersall et al., 2020). Extraction of a membrane protein within a SMALP does not require detergent, and a SMALP is comprised of the lipid bilayer and embedded

protein(s). SMALPs containing the protein of interest can then be purified using affinity tags such as streptavidin. The resulting SMALPs are uniform in size (Morrison et al., 2016) making them ideal candidates for use in solution-based FCS studies (Fig. 2). Limitations of the method are chiefly restricted to the pH and divalent cation sensitivity of the SMALP and also the size limit of the protein to be encapsulated (Lee et al., 2016). Artefacts due to SMALP aggregation are also possible, however when used with FCS, these can be distinguished based on size (Horsey et al., 2020).

The power of combining solution-based FCS with SMALP encapsulated membrane proteins has been demonstrated by two recent publications. In the first, ligand binding to the SMALP purified adenosine A_{2A} receptor (A_{2A}AR) was explored (Grime et al., 2020). The A_{2A}AR is a prototypical member of the GPCR superfamily, and is an attractive drug target for a number of CNS disorders and cancer immunotherapies (Chen et al., 2013). Activation of A_{2A}AR by a ligand leads to coupling to intracellular G-proteins; both processes can be highly dependent on the receptor's conformational organisation stabilised by the plasma membrane (Routledge et al., 2020). In the study by Grime et al., 2020, the A_{2A}AR was expressed in the yeast expression system, *Pichia pastorius*, before being solubilised in SMALP particles from membrane preparations. *Pichia pastorius* is a popular yeast expression system which ensures correct folding and secretion of the recombinant protein alongside a straightforward and expandable extraction process (Singh et al., 2008). Real-time quantification of ligand binding to the SMALP-encapsulated A_{2A}AR was demonstrated through FCS using a fluorescent tracer ligand based on the non-selective adenosine antagonist, xanthine amine congener (Fig. 2(a) and Grime et al., 2020). The key requirement in this study was to demonstrate that the two diffusing species, freely diffusing tracer ligand and receptor-bound tracer ligand, could be separated based on their molecular weights (and hence diffusion co-efficients). One significant advantage of autocorrelation analysis is that functions are additive, which allows the simultaneous quantification of both free and bound tracer ligand in real-time in a homogenous solution. Here, FCS was applied to derive the affinity constant of an unlabelled ligand, ZM241385, and show it was the same as that derived from classic radioligand binding experiments (Fig. 2(b)). This shows the potential of FCS to be exploited to screen unlabelled candidate compounds for affinity, and to derive characteristics, such as binding kinetics, and ultimately calculate drug residency time.

In the second study, the multi-drug ATP binding cassette transporter ABCG2 was SMALP encapsulated to investigate its stoichiometry in membranes (Horsey et al., 2020). As a multi-drug transporter, ABCG2 can transport a wide range of substrates and is also inhibited by a range of molecules including commonly used pharmaceuticals. A single poly-peptide of ABCG2 contains half the domains required for a functional transporter and therefore it is thought that at least two subunits are required to form an active transporter. Previous studies, which include those employing FCS and PCH in live cell membranes, have identified that ABCG2 exists in an oligomeric state (Wong et al., 2016; Taylor et al., 2017). To further investigate the predominant stoichiometry of the transporter, ABCG2 was stably expressed with an N-terminal superfolded GFP-tag (sfGFP) in human embryonic kidney (HEK293T) cells before membrane preparations were subject to SMALP encapsulation. By comparison to monomeric (CD86) and dimeric controls (CD28) (Dorsch et al., 2009), PCH analysis of the encapsulated sfGFP-tagged ABCG2 to measure the molecular brightness of the protein suggested a chiefly homodimeric form (Horsey et al., 2020), demonstrating that the predominant stoichiometry was a true dimer and not higher order oligomers (Fig. 2(c)). The derived diffusion co-efficients from both this study and that by Grime et al., 2020 allowed the SMALP radius to be estimated at 7–9 nm, through application of the Stokes-Einstein equation. Historically, developing a robust binding assay to determine the ability of candidate molecules to ABCG2 has proved challenging (Clark et al., 2006). ABCG2 has inherently low affinity for substrates and using alternative methods such as radio-substrate binding makes the development of an assay to directly screen for substrates and inhibitors difficult. Horsey et al. (2020) took advantage of the availability of a fluorescent derivative of the α_1 adrenergic receptor antagonist, prazosin, which is also a substrate for ABCG2. Specific binding of fluorescent prazosin to SMALP-ABCG2 was measured using FCS (Horsey et al., 2020). As with SMALP encapsulated A_{2A}AR, this method has the potential to be developed into a screening assay to determine the ability of compounds to bind ABCG2 and could allow real-time screening and dynamic evaluation of compound affinity to this multidrug target. The purification of transporter-containing SMALPs was not required in the ABCG2 FCS and PCH stoichiometry experiments (Horsey et al., 2020) and as such ligand-based screening could be simplified by omitting the purification step if used in combination with FCCS (Fig. 2(d)). Doubly-labelled species (representing both receptor and ligand interacting) could be isolated from those emitting due to fluorescent receptor or ligand alone. A similar simplification is seen with the design of a screening assay described by Stoevesandt and Brock (2006). Here FCCS was applied to determine protein interactions in a one-pot assay where cell lysate is combined together with both primary and secondary antibodies with no purification step. Furthermore the assay has been miniaturised to a 384-well format requiring only microliters per assay repeat.

The extraction and subsequent encapsulation of membrane proteins into SMALPs therefore provides a straightforward and widely applicable methodology which can maintain the natural conformation and environment of the target protein within a uniformly sized product. Combining SMALP extraction with FCS and associated applications provides a potentially powerful approach to derive ligand binding affinities and kinetics in a screening format.

6. Conclusions and future directions

The use of FCS and associated techniques (e.g. FCCS, scanning FCS) to study solubilised or extracted membrane proteins is a powerful way to monitor ligand binding and investigate protein stoichiometry with higher throughput and reduced background compared to cell-based studies. None of the techniques described above maintain the true dynamics and cellular regulation of protein function that is found within live cells; cytoskeletal constraint, trafficking and internalisation of receptors, and interacting scaffolding and signalling proteins are all absent. We therefore see solution-based FCS on membrane proteins as complementary to live cell studies. The application of SMALPs to FCS opens a number of future possibilities to its more routine use in drug discovery (Fig. 2). As demonstrated by Antoine et al. (2016), FCS studies can be performed using a multi-well plate-based system and future effort around miniaturisation would allow this to become a relatively high-throughput assay platform. With a view to the future, alternative polymers to SMA have the potential to increase the use of encapsulated proteins in FCS further, displaying reduced auto-fluorescence in the UV range, the ability to tolerate low millimolar concentrations of cations and to dictate the conformational state of the protein (Oluwole et al., 2017; Grime et al., 2021; Harwood et al., 2021). In conclusion, in combination with recent advances in membrane protein purification, solution-based FCS offers a viable route to study ligand binding and stoichiometry of membrane proteins.

CRediT authorship contribution statement

Leigh A Stoddart: Writing – original draft, Writing – review & editing. **Joëlle Goulding:** Writing – original draft, Writing – review & editing, Visualisation. **Stephen J Briddon:** Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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