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Review

Fluorescence-based approaches for monitoring membrane receptor oligomerization

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Membrane protein structures are highly under-represented relative to water-soluble protein structures in the protein data-bank. This is especially the case because membrane proteins represent more than 30% of proteins encoded in the human genome yet contribute to less than 10% of currently known structures (Torres *et al.* in Trends Biol Sci 28:137–144, 2003). Obtaining high-resolution structures of membrane proteins by traditional methods such as NMR and x-ray crystallography is challenging, because membrane proteins are difficult to solubilise, purify and crystallize. Consequently, development of methods to examine protein structure in situ is highly desirable. Fluorescence is highly sensitive to protein structure and dynamics (Lakowicz in Principles of fluorescence spectroscopy, Springer, New York, 2007). This is mainly because of the time a fluorescence probe molecule spends in the excited state. Judicious choice and placement of fluorescent molecule(s) within a protein(s) enables the experimentalist to obtain information at a specific site(s) in the protein (complex) of interest. Moreover, the inherent multi-dimensional nature of fluorescence signals across wavelength, orientation, space and time enables the design of experiments that give direct information on protein structure and dynamics in a biological setting. The purpose of this review is to introduce the reader to approaches to determine oligomeric state or quaternary structure at the cell membrane surface with the ultimate goal of linking the oligomeric state to the biological function. In the first section, we present a brief overview of available methods for determining oligomeric state and compare their advantages and disadvantages. In the second section, we highlight some of the methods developed in our laboratory to address contemporary questions in membrane protein oligomerization. In the third section, we outline our approach to determine the link between protein oligomerization and biological activity.

Keywords. Anisotropy; fluorescence lifetime imaging microscopy; fluorescence; image correlation spectroscopy; membrane; receptor

1. How do we determine the oligomeric state of membrane proteins using fluorescence methods?

Fluorescence is the radiative emission resulting from the transition of an initially excited single state to the ground state. As recognized by Birks, the excited state has chemical and physical characteristics distinct from the ground state. Coupled with the long duration a molecule spends in the excited state, fluorescence is highly sensitive to environment. As a result, experiments can be designed using fluorescence as a probe of structure and dynamics.

Due to the complexity of the biological membranes, determining the oligomeric state of the membrane protein in a cellular environment is challenging.

For the sake of simplicity, we classify fluorescence approaches to determine oligomeric states of membrane proteins into those that infer oligomeric state from dynamics, those that infer oligomeric state from distances and those that infer oligomeric state from brightness.

1.1 *Dynamics (self-diffusion)*

Probably the two most common dynamics parameters are the translational diffusion coefficient and the rotational diffusion coefficient (Lakowicz 2007).

The translational diffusion coefficient measures how fast a molecule diffuses from its initial position. Traditional methods of translational diffusion include fluorescence recovery after photobleaching (FRAP) (Axelrod *et al*. 1976), fluorescence correlation spectroscopy (FCS) (Elson and Magde 1974; Magde *et al*. 1974) and single particle tracking

(SPT) (Kusumi *et al.* 1993). In FRAP, a small region on a membrane is photobleached with an intense laser pulse, and the recovery of unbleached molecules into the pre-bleached region is monitored with a second lower-intensity laser beam. The rate of recovery of the fluorescence is related to the diffusion coefficient and the size of the laser spot. In FCS, spontaneous fluctuations from a small excitation volume are recorded and analysed by a process called autocorrelation. The amplitude and shape of the autocorrelation function delivers information about protein concentration and diffusion. In SPT, a single membrane protein is labelled with a fluorescent or gold particle and movements are tracked in time. A plot of the mean-squared displacement from the average position as a function of time delivers the type of diffusion process (random, constrained or active) and the magnitude of the diffusion coefficient. The slow movement of membrane proteins has garnered the development of the ICS or image correlation spectroscopy (ICS) suite of methods (Peterson *et al*. 1993; Petersen *et al*. 1998). The ICS methods are a boon for the biological community because the instrumentation (confocal or wide-field microscope) is already available in many universities, institutes and hospitals. In temporal ICS (TICS) (Srivastava and Petersen 1996) or space-time ICS (STICS) (Hebert *et al.* 2005), images collected using laser scanning confocal microscopy of labelled membrane proteins on live cells are analysed using correlation methods. Cross-correlation of images collected at different times (TICS) is analogous to FCS and yields information on translational dynamics. In STICS (Hebert *et al*. 2005), the correlation analysis utilizes information from space and time delivering directional and diffusive motion information.

The theoretical work of Saffman and Delbrück (1975) indicated that the translational diffusion coefficient for membrane proteins is not very sensitive to the size of the diffusing protein having a weak logarithmic dependence of lateral diffusion as a function of membrane protein radius. However, recent work suggested the opposite conclusion (Naji *et al.* 2007) with diffusion scaling with the inverse of the particle radius. In one interesting application, some authors have utilised diffusion measurements to measure monomer–dimer equilibria of the unliganded epidermal growth factor receptor on cell surfaces (Chung *et al.* 2010). Nevertheless, the lateral diffusion of membrane proteins can also depend on obstacles in the cell membrane, such as cytoskeletal elements, which provide points of attachment (and ensuing membrane protein immobilization), or "picket fences" that act as corals restricting lateral motions (Kusumi *et al.* 1993).

Rotational diffusion (Lakowicz 2007) measures the rate at which an initially photoselected distribution of excited states reorients to a new orientational distribution. Rotational diffusion for most membrane proteins occurs on the microsecond to millisecond time scales. Therefore, time-resolved polarized fluorescence depletion or polarized fluorescence recovery after photobleaching are used to measure the clustering or organization of membrane proteins (Rahman *et al.* 1992; Yuan and Axelrod 1995) Several proteins have been measured using this method, including Fc receptors and acetylcholine receptors (Rahman *et al.* 1992; Yuan and Axelrod 1995).

According to Saffman and Delbrück (1975), the rotational diffusion coefficient depends on the inverse square of the radius of the membrane protein and is therefore more sensitive to size than the translational diffusion coefficient. It is important to note that inference of membrane protein size, and therefore oligomeric state from dynamics measurements, requires several assumptions such as the (1) theoretical model linking size to dynamics, (2) assumption that the labelled protein is the only protein contributing to the dynamics and (3) assumption that the probe dynamics reflects the protein dynamics.

1.2 *Dynamics (Co-diffusion)*

A very powerful method to detect oligomerization is by measuring the co-diffusion of two proteins, one labelled with a green dye and the other with a red dye. Using cross-correlation fluorescence correlation spectroscopy it is possible to determine the fraction of monomeric-free diffusing complexes and fraction dimerized complexes (Bacia *et al.* 2006). Wohland's laboratory has utilised this approach to measure the fraction of epidermal growth factor receptors in the dimerized state on living cells (Liu *et al.* 2007). The advantage of using co-diffusion is that if two proteins are moving together as a complex then it is likely they are interacting. In contrast, co-localization as defined by green- and red-labelled proteins together in a pixel (or voxel) is of low resolution (ca 250 nm) and cannot distinguish between two proteins in the same region by chance and two proteins that are close together in a complex (figure 1).

1.3 *Distances*

A protein dimer can be defined as two monomers being closely packed together to form a dimeric complex. In this definition, proof of close approach between two or more monomers is evidence for an oligomeric protein. Because an average protein size is around 10 nm in diameter, methods to measure distances on the 1–10 nm scale are required. Fortunately, methods to measure distances in this range are available. Forster resonance energy transfer (or fluorescence resonance energy transfer; FRET) and homo-FRET are two methods to measure separations on the 1–10 nm scale (Lakowicz 2007). In conventional FRET, two probes are used: a donor probe and an acceptor probe. When the donor

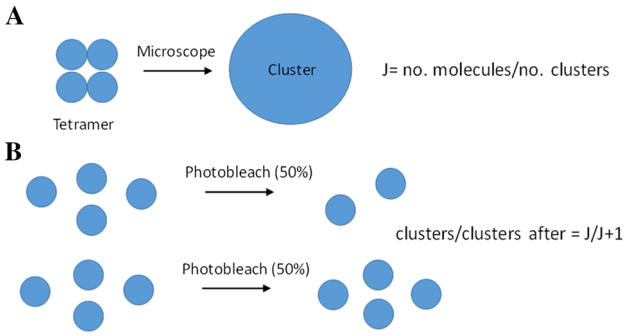

Figure 1. Image correlation spectroscopy and membrane protein oligomerization. (**A**) A conventional microscope blurs the object of a protein oligomer creating an image that appears as a single cluster. By measuring the number or density of clusters and comparing this to the number of proteins (by binding studies or calibrated fluorescence), an average oligomeric state can be determined. (**B**) Photobleaching and oligomeric state. By measuring the relative decrease in number of clusters after bleaching, the oligomeric state of the protein can be determined. Upper example is monomers. In this case, bleaching half the molecules destroys half the clusters. In the lower panel for oligomers, bleaching half the labels does not photodestroy half the clusters.

is excited it can transfer its energy non-radiatively to the acceptor probe. However, this only occurs if the distance between the donor and the acceptor are in the range 1–10 nm and the orientation of the transition moments on the two probes is not orthogonal. The transfer of energy can be detected by several means: (1) reduction of donor fluorescence intensity or lifetime, and (2) increase in sensitized acceptor fluorescence and grow-in of acceptor fluorescence over time (Lakowicz 2007). Evidence of FRET can indicate some form of protein oligomerization on the cell surface; however, the size of the oligomers and the extent of oligomerization are also desirable parameters. To determine oligomeric state, Veatch and Stryer (1977) developed a theoretical formalism relating the size of the protein complex to FRET as a function of labelling ratio. This theory applies to small oligomeric proteins and in the limit where there is no FRET between oligomers. Raicu and Singh (2013) developed an interesting approach using spectral imaging called FRET spectroscopy. In this innovative method, a pixel-by-pixel FRET efficiency is built up and displayed as a histogram. This histogram can then be related to the statistical distribution of distances between labels in an oligomeric membrane protein yielding both distance information and quarternary state information. This approach was applied to a range of membrane proteins, including ABC transporter, VEGF receptor, sigma-1 receptor and the muscarinic M3 acetylcholine receptor (Raicu and Singh 2013; Singh *et al*. 2013; Patowary *et al.* 2013; Mishra *et al.* 2015).

In the homo-FRET approach, a fluorescent probe with a small Stokes shift is used. A probe with a small Stokes shift has sufficient overlap between absorption and emission to act as both an energy transfer donor and an energy transfer acceptor (Lakowicz 2007). Therefore, homo-FRET is more sensitive than conventional FRET. However, unlike conventional FRET, homo-FRET does not change the intensity or the excited-state lifetime of the probe. Homo-FRET results in the reduction of the emission polarization anisotropy of the probe (Lakowicz 2007). The emission polarization (or anisotropy) is a measure of how the orientational distribution of the emitting states changes after initial photoselection (Lakowicz 2007). An important caveat is that emission polarization can be influenced by protein motions as well as homo-FRET. Therefore, the best way to detect homo-FRET between two or more proteins is by varying the extent of probe labelling. This can be done by mixing labelled and unlabelled proteins in solution or alternatively by photobleaching. Major's laboratory was the first to utilize homo-FRET with photobleaching and they used it to determine the clustering GPI-linked proteins into rafts (Varma and Mayor 1998). We, as well as other researchers, have developed a theoretical formalism to determine the oligomeric state of a membrane protein and the presence of higher-order oligomers (Yeow and Clayton 2007). With this approach, we determined that the unliganded epidermal growth factor receptor is in 90% dimers and 10% monomers on BaF/3 cells, and a truncated form of the epidermal growth factor receptor (found in brain cancer) is in oligomers on BaF/3 cells (Kozer *et al.* 2011). When applied to the serotonin receptor, evidence for higher-order oligomers was found, which was dependent on membrane environment (Ganguly *et al.* 2011).

While hetero- and homo-FRET measurements are an appealing way to determine oligomer states and sizes, there are some crucial assumptions and caveats surrounding these methods. First, the high density of expression of some membrane proteins can lead to high levels of membrane protein at the cell membrane. Under these circumstances, the average distance between monomeric proteins can approach the Forster distance for energy transfer and give rise to by-stander FRET effects. In other words, FRET when there is no oligomerization-only crowding. Kenworthy and Edidin (1998) provided a framework for detecting by-stander FRET in the past. More recently, Hristova's laboratory demonstrated, both theoretically and experimentally, how protein expression level influences FRET between oligomers (King *et al*. 2014). The 'take-home message' from these studies is that FRET studies should be performed when cell expression levels of the membrane protein of interest are 50,000 or less proteins per cell. At this lower level, random by-stander effects can be considered negligible. If protein expression is higher, then corrections should be made (King *et al*. 2014; King *et al*. 2016). Moreover, if probes are located more than twice the Forster radius apart, then FRET will be too small to be detected and information about the separation (and possibly the oligomeric state) is lost. Here, super-resolution

methods can potentially help but the scope of this method is beyond this review article. The reader is referred to the excellent work of Martin-Fernandez and colleagues, who are pushing the limits of super-resolution to within the FRET range (for further information see Needham *et al.* 2016 and references therein).

1.4 *Brightness*

If we consider a fluorescent probe to have certain brightness, analogous to a small light bulb, then two probes will be twice as bright, three probes thrice as bright and so on. Brightness of labelled membrane proteins can be inferred by several methods. Photon counting histogram method (Chen *et al.* 1999) utilizes the photon stream from single complexes as they move into a focused region. Statistical analysis of the photon counts yields a photon count histogram, which is related to the average brightness of the moving complexes. Number and brightness (Digman *et al.* 2008) is another method that uses images from a confocal microscope to determine concentration and brightness of diffusing molecules. An important caveat in this method is that any immobilised molecule has a brightness of 1. Fluorescence intensity distribution analysis (Godin *et al.* 2011) is yet another method for determining brightness. This method can be applied to fixed or live cells. It has evolved to determine oligomeric states of membrane proteins in different organelles of the cell, i.e. at sub-cellular distribution, and complex models of oligomerization, i.e. involving more than one type of oligomeric state. A caveat from these approaches is that brightness is referenced to a monomeric fluorophore, which may or may not have the same properties (photophysics or labelling) as observed inside living cells. This can render absolute quantification of oligomeric state and oligomeric state distribution problematic.

Single molecule methods count the number of bleaching steps to complete photodestruction. The number of steps indicates the number of fluorophores per imaged complex and therefore the oligomeric state (Ulbrich and Isacoff 2007). Isacoff and colleagues adopted this method in several membrane proteins expressed on the surface of oocytes (Ulbrich and Isacoff 2007). The large area of the membrane on these cells and controllable expression protocols enables the examination of membrane protein complexes at very low surface densities. Importantly, since the single molecule photobleaching approach relies only on a number of bleaching steps, it does not require a brightness standard.

Earlier research has employed several methods to delineate the dynamics and oligomerization of a membrane protein (Crosby *et al.* 2013). Gadella's group exploited a range of dynamic approaches including FRET, FRAP, FCS and photon-counting histogram for in-depth understanding of membrane protein dynamics and interactions on a range of length and time scales (Crosby *et al.* 2013).

2. Image correlation methods for determining the oligomeric state of membrane proteins developed in our laboratory

2.1 *Concept*

To examine the membrane protein of interest, we must first label it with a fluorophore (typically either GFP-type or fluorophore-ligand or fluorophore-antibody conjugate) and check whether the protein is functional. Suppose we label each monomer of a membrane protein oligomer with a fluorophore. The simplest method to determine the quaternary state of a membrane protein would be to just image it at the membrane with a fluorescence microscope and visually count the number of sub-units (monomers). However, because of the diffraction of light, the density of membrane proteins and low signal to noise, this is not possible. With standard confocal fluorescence microscopy, a single oligomer will appear as one spot in fluorescence, if visible at all above the noise. For example, a tetramer will appear as a single blob or cluster of fluorescence and not as four resolved single spots. However, we can overcome these problems in several ways.

(1) Image correlation spectroscopy and cluster density: we can count the number of clusters instead of the number of fluorophores per cluster. Initially this appears to be wrong, but let us explore how this works.

(a) If the total number of clusters per cell and the number of membrane proteins per cell (from binding studies or average fluorescence signal, for example) are known, we can determine the average number of membrane proteins per cluster. The oligomer size, J, is given by

J = number of molecules per cell/number of clusters per cell

Determining the number of clusters from an image of a cell surface is with an image analysis technique called image correlation spectroscopy. This method recognizes an image of fluorescence as a record of fluctuations in space. The image correlation approach generates a spatial autocorrelation function image, which ultimately extracts the cluster density (number of clusters per area). The image of fluorescence can be obtained using relatively simple instrumentation—either confocal microscope or wide-field microscope—and with standard labels such as GFP or fluorophore-conjugated antibodies.

Nils Petersen and Paul Wiseman were the first to develop this method and utilize it to determine oligomeric sizes of a number of cell surface receptors (Peterson *et al.* 1993). Platelet-derived growth factor receptors clustered as tetramers on cells, while epidermal growth factor receptor was highly clustered in groups of 10–30 receptors per cluster on cancer cells (Petersen *et al.* 1998). We utilized this method to determine a ligand-induced dimer to tetramer transition of epidermal growth factor receptor in normal cells (Clayton

et al. 2005; Kozer *et al.* 2013) and to determine the influence of therapeutic antibodies on clustering of cell surface receptors (Kozer *et al.* 2011).

(b) If we do not know the number of membrane proteins per cell, we can use photobleaching to destroy half the fluorescent labels. By comparing the number of clusters per cell before bleaching and those after photobleaching, we can infer the oligomeric state.

Suppose we have a monomeric protein on the cell surface and the number of clusters is B per cell. Then we bleach half the number of fluorophores. Because there is one fluorophore per clusters, we will have B/2 clusters per cell.

Now suppose the oligomeric state is a dimer and we have B clusters per cell, each containing two labels. If we bleach half the labels, we now have B/4 dimers with two fluorophores, B/2 dimers with one fluorophore and B/4 dimers with no labels. That is to say, there are now more than half of labelled clusters remaining even though half the labels are bleached.

When we use image correlation with photobleaching we refer to this technique as photobleaching image correlation spectroscopy (Ciccotosto *et al.* 2013).

In general, if the number of labelled clusters per cell before bleaching is B, then the number of labelled clusters after bleaching half the fluorophores is $B(J/(J + 1)$, where J is the oligomeric state.

With photobleaching image correlation, one can also determine the oligomer size distribution (Lajevardipour *et al.* 2015). In this case, one needs to determine the cluster density as a function of photobleaching for a series of different photobleaching extents. In our first implementation of this method (Ciccotosto *et al.* 2013), we revealed time-dependent increases in A peptide oligomerization on neuronal cell cultures. Although no definitive model could be extracted from the data, we showed that the oligomerization extended from monomers, dimers, and so on, to pentamers or hexamers over time. We have also utilized this approach to show clustering of epidermal growth factor over a range of oligomeric sizes on CHO cells. This work complements the use of super-resolution techniques that reveal distances between labelled ligands consistent with the presence of extended linear oligomers on Chinese Hampster Ovary (CHO) cells (Needham *et al.* 2016). More recently, in collaboration with Prof Chattopadhyay, we have determined the monomer–dimer–trimer distribution of serotonin receptor on clustered cells and the influence of membrane cholesterol on this distribution (Chakraborty *et al.* 2018). This work is in progress.

3. How do we determine the link between oligomeric state of a membrane protein and biological activity?

The fluorescence lifetime is sensitive to environment (Berezin and Achilefu 2011) and when combined with Forster resonance energy transfer (FRET) (Martin-Fernandez *et al.* 2000), it is a useful probe of membrane protein activation. For many systems protein, activation results in either phosphorylation or recruitment of specific proteins to the protein of interest. By tagging an anti-phosphotyrosine antibody or the protein interaction partner with a suitable energy transfer acceptor, one can detect membrane protein activation through a change in the fluorescence lifetime of a donor-labelled membrane protein. Because FRET is sensitive to the inverse sixth power of separation and operative over the 2–10 nm scale, intimate interactions are detected alone. Fluorescence lifetime imaging is not as common as fluorescence imaging but increasingly made available due to commercial production. In our laboratory, we do fluorescence lifetime imaging in the frequency domain, which means we detect a phase delay of fluorescence relative to a sinusoidally modulated excitation. We made use of an already-developed FLIM-FRET assay for measuring epidermal growth factor receptor phosphorylation (activation) and combined this assay with ICS (i.e. FLIM-ICS) to determine the cluster densities and relative sizes of phosphorylated and unphosphorylated epidermal growth factor receptor (Clayton *et al.* 2008). From FLIM images containing a GFP-tagged epidermal growth factor receptor and an anti-phosphotyrosine conjugated to AlexaFluor555, we were able deconvolute the fluorescence of the activated epidermal growth factor receptor from the inactive epidermal growth factor receptor. This is because the lifetimes of the GFP tag are different in the two states and the frequency responses are different. From the optically separated images of the active and inactive epidermal growth factor receptor, we were able to determine the fraction of phosphorylated epidermal growth factor receptors, fraction of unphosphorylated epidermal growth factor receptors, the cluster density of phosphorylated epidermal growth factor receptors, the cluster density of unphosphorylated epidermal growth factor receptors and, importantly, the relative brightness (or cluster size) of the activated epidermal growth factor receptor relative to the unactivated epidermal growth factor receptor. These quantitative parameters were compared to different models for the epidermal growth factor receptor activation process. For a monomer to a dimer transition model, the relative brightness of activated epidermal growth factor receptor to unactivated epidermal growth factor receptor has a maximum value of two. For a dimer conformational change model, the relative brightness of activated to unactivated epidermal growth factor receptor is 1. Our measurements revealed that the relative brightness was 4. This result was more consistent with a monomer–dimer–tetramer model, i.e. involving a pre-equilibrium between monomer–dimer states in the absence of ligand and a ligand-induced dimer to tetramer transition. An alternative model involving dimer–dimer polymerization reaction was also considered and compatible with the available data. These results showed that active dimers were not the only active epidermal growth

factor receptor species and that epidermal growth factor receptor tetramers and higher-order oligomers were also phosphorylated as a major species (Clayton *et al.* 2008). We later extended this work to examine adaptor recruitment to the epidermal growth factor receptor and revealed very similar results (Kozer *et al.* 2014). In other words, tetramers and higher-order oligomers appear to be associated with full adaptor recruitment to the receptor (Kozer *et al.* 2014). Biophysical studies emphasized the importance of linking measurements with models. Collaborating with experts in rule-based modelling (Richard Posner (Clayton *et al.* 2008) and Bill Hlavacek (Kozer *et al.* 2013; Kozer *et al.* 2014) enabled us to go beyond observations of average oligomeric state and build actual predictive models of the oligomerization processes. In this respect, developments in theory and experiment should go hand in hand to produce a more comprehensive understanding of oligomerization phenomena *in situ*.

4. Concluding remarks

We have attempted to provide a select overview of fluorescence approaches to determine oligomeric states of membrane proteins. We have discussed the advantages and disadvantages of the techniques in relation to whether the technique derives dynamics, proximity or brightness information.

Particularly for the biology community, we espouse the benefits of analysing fluorescence images by correlation obtained with standard confocal microscopy to determine cluster densities and cluster sizes of fluorescently tagged membrane proteins. We believe these methods are the most accessible to the biology community. By making use of the natural photobleaching that occurs with replicate image acquisition, one can even determine the aggregation distributions of membrane proteins. Applying similar analyses to fluorescence lifetime images extends the image correlation approach to biological function, providing the possibility to directly link membrane protein quaternary state to membrane protein function.

References

Axelrod D, Koppel D, Schlessinger J, Elson E and Webb W 1976 Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* **16** 1055–1069

Bacia K, Kim SA and Schwille P 2006 Fluorescence cross-correlation spectroscopy in living cells. *Nature Methods* **3** 83–89

Berezin MY and Achilefu S 2011 Fluorescence lifetime measurements and biological imaging. *Chem. Rev.* **110** 2641–2684

Chakraborty H, Jafurulla Md, Clayton AHA and Chattopadhyay A 2018 Exploring oligomeric state of the serotonin1A receptor utilizing photobleaching image correlation spectroscopy: implications for receptor function. *Faraday Discuss*. **207** 409–421

Chen Y, Müller JD, So PT and Gratton E 1999 The photon counting histogram in fluorescence fluctuation spectroscopy. *Biophys. J.* **77** 553–567

Chung I, Akita R, Vandlen R, Toomre D, Schlessinger J and Mellman I 2010 Spatial control of EGF receptor activation by reversible dimerization on living cells. *Nature* **464** 783–787

Ciccotosto GD, Kozer N, Chow TT, Chon JW and Clayton AH 2013 Aggregation distributions on cells determined by photobleaching image correlation spectroscopy. *Biophys. J.* **104** 1056–1064

Clayton AH, Orchard SG, Nice EC, Posner RG and Burgess AW 2008 Predominance of activated EGFR higher-order oligomers on the cell surface. *Growth Factors* **26** 316–324

Clayton AH, Walker F, Orchard SG, Henderson C, Fuchs D, Rothacker J, Nice EC and Burgess AW 2005 Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor–a multidimensional microscopy analysis. *J. Biol. Chem.* **280** 30392–30399

Crosby KC, Postma M, Hink MA, Zeelenberg CH, Adjobo-Hermans MJ and Gadella TW 2013 Quantitative analysis of self-association and mobility of annexin A4 at the plasma membrane. *Biophys. J.* **104** 1875–1885

Digman MA, Dalal R, Horwitz AF and Gratton E 2008 Mapping the number of molecules and brightness in the laser scanning microscope. *Biophys. J.* **94** 2320–2332

Elson EL and Magde D 1974 Fluorescence correlation spectroscopy I. Conceptual basis and theory. *Biopolymers* **13** 1–27

Ganguly S, Clayton AH and Chattopadhyay A 2011 Organization of higher-order oligomers of the serotonin1(A) receptor explored utilizing homo-FRET in live cells. *Biophys. J.* **100** 361–368

Godin AG, Costantino S, Lorenzo LE, Swift JL, Sergeev M, Ribeiro-da-Silva A, De Koninck Y and Wiseman PW 2011 Revealing protein oligomerization and densities in situ using spatial intensity distribution analysis. *Proc. Natl. Acad. Sci. USA* **108** 7010–7015

Hebert B, Costantino S and Wiseman PW 2005 Spatiotemporal image correlation spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO cells. *Biophys. J.* **88** 3601–3614

Kenworthy AK and Edidin M 1998 Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 Å using imaging fluorescence resonance energy transfer. *J. Cell Biol.* **142** 69–84

King C, Sarabipour S, Byrne P, Leahy DJ and Hristova K 2014 The FRET signatures of noninteracting proteins in membranes: simulations and experiments. *Biophys. J.* **106** 1309–1317

King C, Stoneman M, Raicu V and Hristova K 2016 Fully quantified spectral imaging reveals in vivo membrane protein interactions. *Integr. Biol.* **8** 216–229

Kozer N, Barua D, Henderson C, Nice EC, Burgess AW, Hlavacek WS and Clayton AH 2014 Recruitment of the adaptor protein Grb2 to EGFR tetramers. *Biochemistry* **53** 2594–2604

Kozer N, Barua D, Orchard S, Nice EC, Burgess AW, Hlavacek WS and Clayton AH 2013 Exploring higher-order EGFR oligomerisation and phosphorylation—a combined experimental and theoretical approach. *Mol. Biosyst.* **9** 1849–1863

Kozer N, Kelly MP, Orchard S, Burgess AW, Scott AM and Clayton AH 2011 Differential and synergistic effects of epidermal growth factor receptor antibodies on unliganded ErbB dimers and oligomers. *Biochemistry* **50** 3581–3590

Kusumi A, Sako, Y and Yamamoto, M 1993 Confined lateral diffusion of membrane receptors as studied by single particle tracking (Nanovid Microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys. J.* **65** 2021–2040

Lajevardipour A, Chon JWM and Clayton AHA 2015 Determining complex aggregate distributions of macromolecules using photobleaching image correlation microscopy. *AIMS Biophys.* **2** 1–7

Lakowicz JR (2007) Principles of fluorescence spectroscopy, 3rd Edition. Springer, New York

Liu P, Sudhaharan T, Koh RM, Hwang LC, Ahmed S, Maruyama IN and Wohland T 2007 Investigation of the dimerization of proteins from the epidermal growth factor receptor family by single wavelength fluorescence cross-correlation spectroscopy. *Biophys. J.* **93** 684–698

Magde D, Elson EL and Webb WW 1974 Fluorescence correlation spectroscopy II. An experimental realization. *Biopolymers* **13** 29–61

Martin-Fernandez ML, Clarke DT, Tobin MJ, and Jones GR 2000 Real-time studies of the interactions between epidermal growth factor and its receptor during endocytic trafficking. *Cell. Mol. Biol*. **46** 1103–1112

Mishra AK, Mavlyutov T, Singh DR, Biener G, Yang J, Oliver JA, Ruoho A and Raicu V 2015 The sigma-1 receptors are present in monomeric and oligomeric forms in living cells in the presence and absence of ligands. *Biochem. J.* **466** 263–271

Naji A, Levine AJ and Pincus PA 2007 Corrections to the Saffman–Delbrück mobility for membrane bound proteins. *Biophys. J.* **93(11)** L49–L51

Needham SR, Roberts SK, Arkhipov A, Mysore VP, Tynan CJ, Zanetti-Domingues LC, Kim ET, Losasso V, Korovesis D, Hirsch M, Rolfe DJ, Clarke DT, Winn MD, Lajevardipour A, Clayton AH, Pike LJ, Perani M, Parker PJ, Shan Y, Shaw DE and Martin-Fernandez ML 2016 EGFR oligomerization organizes kinase-active dimers into competent signalling platforms. *Nat. Commun*. **7** 13307

Patowary S, Alvarez-Curto E, Xu TR, Holz JD, Oliver JA, Milligan G and Raicu V 2013 The muscarinic M3 acetylcholine receptor exists as two differently sized complexes at the plasma membrane. *Biochem. J.* **452** 303–312

Petersen NO, Brown C, Kaminski A, Rocheleau J, Srivastava M and Wiseman PW 1998 Analysis of membrane protein cluster densities and sizes in situ by image correlation spectroscopy. *Faraday Discuss*. **111** 289–305; (discussion 331–343)

Peterson NO, Wiseman PW, Seger O, and Magnusson KE 1993 Quantitation of membrane receptor distributions by image correlation spectroscopy: concept and application. *Biophys. J.* **65** 1135–1146

Rahman NA, Pecht I, Roess DA and Barisas BG 1992 Rotational dynamics of type I Fc epsilon receptors on individually-selected rat mast cells studied by polarized fluorescence depletion. *Biophys. J.* **61** 334–346

Raicu V and Singh DR 2013 FRET spectrometry: a new tool for the determination of protein quaternary structure in living cells. *Biophys. J.* **105** 1937–1945

Saffman PG and Delbrück M 1975 Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA* **1972** 3111–3113

Singh DR, Mohammad MM, Patowary S, Stoneman MR, Oliver JA, Movileanu L and Raicu V 2013 Determination of the quaternary structure of a bacterial ATP-binding cassette (ABC) transporter in living cells. *Integr. Biol*. **5** 312–323

Srivastava M and Petersen NO 1996 Image cross-correlation spectroscopy: a new experimental biophysical approach to measurement of slow diffusion of fluorescent molecules. *Methods Cell Sci*. **18** 47–54

Torres J, Stevens TJ and Samsó M 2003 Membrane proteins: the 'Wild West' of structural biology. *Trends Biol. Sci.* **28** 137–144

Ulbrich MH and Isacoff EY 2007 Subunit counting in membrane-bound proteins. *Nat. Methods* **4** 319–321

Varma R and Mayor S 1998 GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* **394** 798–801

Veatch W and Stryer L 1977 The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol*. **113** 89–102

Yeow EK and Clayton AH 2007 Enumeration of oligomerization states of membrane proteins in living cells by homo-FRET spectroscopy and microscopy: theory and application. *Biophys. J.* **92** 3098–3104

Yuan Y and Axelrod D 1995 Subnanosecond polarized fluorescence photobleaching: rotational diffusion of acetylcholine receptors on developing muscle cells. *Biophys. J.* **69** 690–700