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Resolving Membrane Protein–Protein Interactions in Live Cells with Pulsed Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy

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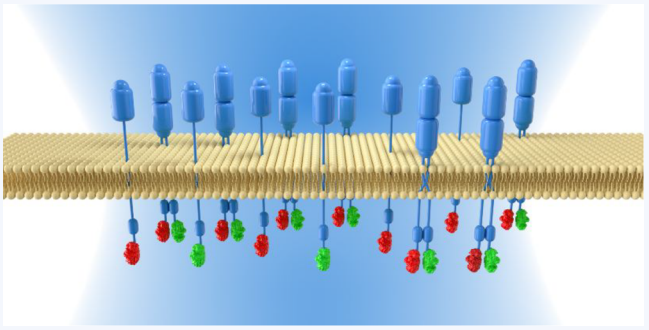
CONSPECTUS: The cell plasma membrane (PM) contains thousands of proteins that sense and respond to the outside environment. These proteins have evolved sensitivity to a wide variety of physical and chemical signals and act as a delivery system across the PM. Membrane proteins are critical for information flow and decision making in the cell and thus are important targets in drug development. A critical aspect of membrane protein function is the way they interact with other proteins, often through the formation of dimers or small oligomers that regulate function at the protein, cell, and organism levels. Resolving membrane protein interactions in a live cell environment is challenging because of the chemical diversity and spatial heterogeneity of the PM. In this Account, we describe a fluorescence technique called pulsed interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) that is ideally suited to quantify membrane associations in live cells. PIE-FCCS is a two-color fluorescence fluctuation method that can simultaneously measure the concentration, mobility, proximity, and oligomerization state of membrane proteins in situ. It has several advantages over two related approaches, single-molecule tracking (SMT) and Förster resonance energy transfer (FRET), including that it measures all of the properties listed above in a single measurement. Another advantage is that PIE-FCCS is most sensitive at the physiological expression levels for many membrane proteins rather than the very low or high levels typical in other techniques. Here, we review the history of FCCS as it has been applied to study membrane protein interactions in cells. We also describe PIE-FCCS and the advantages it has over biochemical approaches like coimmunoprecipitation (co-IP) and proximity ligation assays (PLA). Finally, we review two classes of membrane proteins that have been studied with FCCS and PIE-FCCS: receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). For RTKs, ligand induced dimerization directly regulates the catalytic activity of the kinase, but higher order oligomerization and ligand-independent dimerization can complicate this historically simple paradigm. PIE-FCCS data have resolved a low population of EGFR dimers under basal conditions and assembly into multimers when stimulated with ligand. While GPCRs function primarily as monomers, dimerization has been hypothesized to regulate function for some receptors. PIE-FCCS data have established the dimerization potential of rhodopsin at low densities and were critical for the discovery of a novel dimerization interface in human cone opsins. This Account describes the how FCCS and PIE-FCCS can reveal the details of quaternary interactions in each of these receptor systems.



INTRODUCTION

Membrane protein–protein interactions directly regulate cell communication. These interactions range from high affinity covalent interactions to low affinity, transient complexes. Quantifying these interactions, however, is still very challenging. Why is this? First, membrane proteins are difficult to express and purify using recombinant protein expression tools. They have evolved to be stable in a membrane environment and can only be solubilized in water with carefully selected detergents. Second, the lipid composition of the PM is complex and heterogeneous with dynamic assembly and organization that has yet to be fully characterized. This dynamic chemical heterogeneity makes it difficult to reliably extrapolate the results of in vitro model membrane assays to living systems. Detergent solubilization and model membranes may also negatively affect membrane protein function. So, despite the many excellent in vitro methods for determining protein binding affinities, it remains necessary to test hypothetical membrane protein interactions in live cells. The

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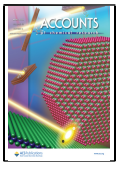

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subject of this article is a time-resolved, single-molecule approach called pulsed interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) that can quantify membrane protein dimerization and oligomerization in live cells.[1]

Membrane proteins have evolved a complex network of interactions with the extracellular environment, lipids, and other proteins in the PM and cytoplasm. One example that will be discussed below is the epidermal growth factor receptor (EGFR). EGFR is a receptor tyrosine kinase (RTK) that drives cell growth and morphology and is the target of new anticancer medications. The assembly of EGFR into dimers and small oligomers directly regulates its function, and ligand induced dimerization is the canonical model for EGFR activation.[2] However, depending on cell type, expression level, and ligand−receptor combinations, ligand binding can either induce dimerization, stabilize preexisting dimers, generate higher order oligomerization, or modulate heterodimerization with other membrane proteins. Because of the multiplicity of interactions, there is a clear need to quantitatively resolve the degree of EGFR oligomerization in situ and determine how the interactions affect receptor function. Such measurements are still very challenging, and the requisite level of quantitative detail has only been achieved with a few well-studied membrane proteins. There is still an enormous interaction space that has not been investigated with live-cell biophysical methods.

The most common tests of membrane protein associations are biochemical methods such as co-IP or PLA. Co-IP detects protein−protein interactions that survive the lysis, elution, and wash steps, and is therefore not ideal for detecting low affinity binding.[3] Furthermore, the selection of detergent/surfactant for stabilizing membrane proteins may affect the results by destabilizing native interactions or stabilizing non-native interactions. The PLA approach is to cross-link the analytes in situ and then analyze the complexes after isolation.[4] This method may detect native interactions but may also trap transient complexes and is thus dependent on the conditions of the assay. Co-IP and PLA are valuable tools for screening large sets of possible interactions, but more quantitative methods are needed to access molecular associations in cells.

Fluorescence-based biophysical assays can measure molecular interactions in live cells because of their biological compatibility and high signal-to-noise contrast. The spatial resolution of these assays is hundreds of nanometers, so straightforward imaging and analysis cannot resolve protein−protein interactions at the level of monomers, dimers and small oligomers. Single molecule imaging methods have been developed to localize single particles and count monomer and dimer populations. The main drawback of these localization methods is that they require the particle spacing to be larger than the diffraction limit, which means the concentration needs to be very low (<1 molecules/μm^2).[5] This is prohibitive for many membrane proteins whose physiological expression level is often above 10−100 molecules/μm^2. Stochastic activation methods were developed to access higher concentration regimes while maintaining high resolution.[6] The stochasticity, however, creates a fundamental problem with measuring dimers or small oligomers because after one of the protomers is localized, it can diffuse to another position before the second protomer is localized. One solution to this problem is cell fixation to immobilize the particles, but this has the potential to stabilize non-native interactions or destabilize native interactions in the same way as the PLA and co-IP methods above.

Energy transfer methods like Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) can detect protein−protein interactions on the order of 5 nm. These are two-color assays that can be imaged and analyzed in many different ways. FRET data, for example, can be collected with single-molecule tracking (SMT) at concentrations less than 1 molecules/μm^2, or much more commonly at bulk densities in the 10^4 to 10^6 molecules/μm^2 range. At high densities, the concentration is not measured directly but is calibrated with an intensity comparison method. FRET data are also sensitive to transient collisions, which become more frequent at high density, making it susceptible to false-positive identification of dimerization.

PIE-FCCS is a time-domain spectroscopy that measures correlated diffusion events at the single-molecule level. It is directly sensitive to stable molecular associations at physiologically relevant concentrations of 10^1−10^3 molecules/μm^2. As a time-correlated single-photon counting (TCSPC) technique, it is also capable of measuring fluorescence lifetimes, and thus, PIE-FCCS combines the advantages of FRET with those of FCCS. In this paper, we review how FCCS and PIE-FCCS have been applied to study membrane protein interactions. There has been significant growth in this area, and we highlight recent work from other laboratories as well as our own to study the assembly and organization of both single- and multipass transmembrane proteins.

BACKGROUND AND DEVELOPMENT OF PIE-FCCS

Fluorescence (Cross-) Correlation Spectroscopy

FCCS is one of several methods based on fluorescence fluctuation spectroscopy (FFS).[7] In FFS methods, time-dependent fluctuations in fluorescence intensity are used to quantify molecular properties like motion, local number density, and molecular brightness. The core idea behind FFS is that fluctuations from a well-defined region of the sample depend directly on changes in the molecular density. In other words, as molecules diffuse into (or out of) a region of interest, they contribute to a net increase (or decrease) of fluorescence signal. This effect is optimized at low concentrations and small regions of interest, where single molecules diffusing in and out of the region will have a significant effect on the signal. Most applications of FFS rely on confocal detection geometries that limit the region of interest to femtoliter volumes.[8]

In single-color FCS, intensity fluctuations are analyzed with a time−time autocorrelation function (ACF), and the results are plotted on a semilog axis to better visualize the range of time scales (Figure 1). Because the focus of this paper is on membrane systems, equations will be discussed for 2D diffusion. The ACF is modeled by an algebraic function, $G(\tau) = G(0)/(1 + \tau/\tau_D)$, with two free parameters: the initial amplitude, $G(0)$, and the decay time, τ_D. The initial amplitude of the ACF reflects the size of the fluctuations and is inversely related to the average number of molecules in the observation area (Figure 1A). The decay time is referred to as the dwell time because it reflects the average time spent by single molecules in the observation area (Figure 1B). If the size of the observation area is calibrated, the number of molecules can be converted to a molecular density or concentration and the dwell time can be converted to a diffusion coefficient. Typical membrane densities accessible in FCS experiments are

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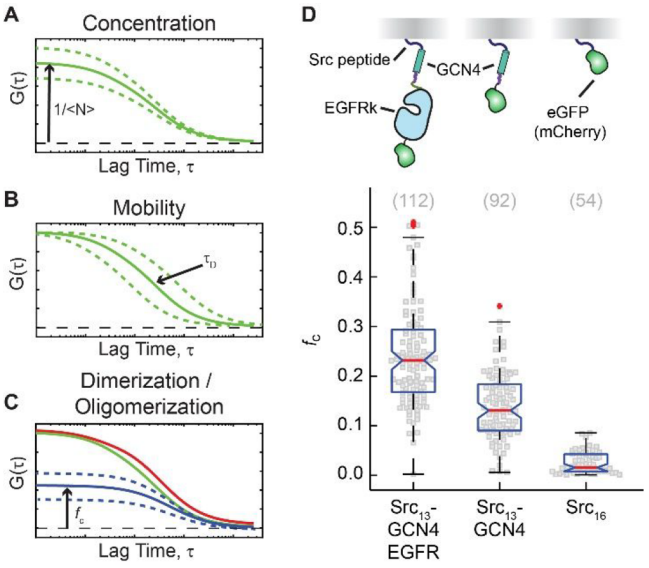

Figure 1. Summary of the information available in FCCS data. (A) The amplitude of each ACF is inversely proportional to the average number of the diffusing species, $\langle N \rangle$. From this parameter, it is possible to calculate the receptor density if the size of the detection area has been calibrated. (B) The decay time of each ACF, τ_D, is the average dwell time of the diffusing species. From this parameter, one can calculate the diffusion coefficient, $D = \omega^2/4\tau_D$. (C) The amplitude of the CCF is related to the fraction of receptors in a complex, f_c, and is sensitive to the distribution of monomers, dimers, and small oligomers. (D) Sample data showing the f_c distributions from single-cell FCCS measurements of a monomer (Src_{16}), dimer (Src_{13}-GCN4), and multimer (Src_{13}-GCN4-EGFR) control. Panel D was reproduced with permission from ref 9. Copyright 2016 eLife.

between 10 and 1000 molecules/μm^2, while accessible membrane protein diffusion coefficients range from 0.05 to 2 $\mu m^2/s$. If the mobility is below this range, methods like single-particle tracking are better suited. Thus, FCS data can be used to monitor concentration and diffusion with quantitative accuracy.

In dual-color FCCS, the system is labeled with two spectrally distinct fluorophores. Emission from the two fluorescent probes is separated so that each corresponding signal can be analyzed independently. This means that both populations have a corresponding ACF, and the respective molecular density and diffusion coefficients can be determined independently. This is an advantage of FCCS compared to related methods like FRET, which do not directly measure diffusion and molecular density. In FCCS, the emission signals from both color channels are also used to calculate a cross-correlation function (CCF) (Figure 1C). The amplitude and decay time of the CCF reflect the density and mobility of diffusing oligomers that contain both fluorescent probes. The amplitude of the CCF is directly sensitive to molecular assemblies that are stable as they move through the detection area. This sensitivity to stable interactions is a unique aspect of FCCS compared to FRET methods, where energy transfer can occur during transient collisions.

First Applications of FCCS to Membrane Proteins

The first application of FCCS to study membrane protein interactions in cells was in 2002.[10] The A and B subunits of bacterial cholera toxin (CTX) were labeled with spectrally resolvable fluorescent dyes (Cy2 and Cy5). The mixture was introduced to live Vero cells, where the CTX subunits associated with the PM. The FCCS data showed that the A and B subunits of the CTX complex were bound at the PM during endocytosis. The study was an important demonstration that membrane associated protein−protein interactions could be resolved with FCCS. However, the low mobility of CTX led to significant complications with the correlation analysis.

The next study of membrane protein interactions in cells was presented in an investigation of interleukin (IL) receptor organization in T cells.[11] Fluorescence labeling was achieved with antibodies against IL-15Rα, IL-2Rα, and MHC I and measurements were made in human Kit 225 lymphoma cell lines. The FCCS data showed that the diffusion of the receptors was correlated, which was interpreted as evidence for stable receptor association. More recent FCCS work resolved the 2D binding affinity of the IL-4Rα/IL-13Rα1 hetero-complexes upon stimulation with IL-4 or IL-13.[12] A 2016 study showed how the coclustering of IL-2Rα and IL-15Rα was regulated by the expression level of MHC I.[13] Several other immune receptors have been studied with FCCS like CNTF receptors, T cell receptors, and adaptor proteins like LAT, but due to space limitations they will not be discussed here.[14−17]

PIE-FCCS Background

Early studies of membrane protein organization with FCCS showed a promising array of new insight, but the difficulty of data collection and analysis has limited the range of applications. PIE is an alternative method for FCCS data collection, where two pulsed lasers are phase delayed so that the emission photon arrival times can be assigned to a specific laser pulse.[1] This allows for spectral cross-talk removal, which is particularly valuable for membrane proteins because of their low mobility and heterogeneous organization. In fact, a forerunner of PIE-FCCS was first applied to the membrane protein Connexin 46, a hexameric gap junction protein.[18] The first PIE-FCCS experiment was reported by Lamb et al. in 2005.[1] Rather than repeat all of the experimental details, we will outline the essential features of PIE-FCCS experiments and highlight key concepts for interpreting the data.

The first requirement for PIE-FCCS experiments is the use of high repetition rate laser sources instead of continuous wave lasers. In our current system, for example, an 80 MHz laser is reduced to 10 MHz with a pulse picker so that there is 100 ns between each pulse. The laser pulse duration should be substantially smaller than the fluorescent lifetime of the dyes that are being probed. This feature makes it possible to record fluorescent lifetime histograms, which are sensitive to FRET and other environmental factors like pH, hydrophobicity, and oxygen levels. The second requirement for PIE-FCCS is that the two pulsed lasers are synchronized in time and then phase delayed by approximately 50 ns. This allows enough time for common fluorescent proteins like eGFP (2.6 ns) and mCherry (4.2 ns) to relax to their ground state before the arrival of another laser pulse. The third requirement for PIE-FCCS experiments is that the data be recorded with TCSPC so that the raw photon data can be time-gated prior to analysis.

To process the PIE data, two time-gates are created to isolate photons arriving after each excitation pulse (Figure 2). The time-gated photons are binned to create the intensity traces ($I(t)$). For a two-color experiment there are two time-gates (0 and 1) and two detection channels (green, G, and red,

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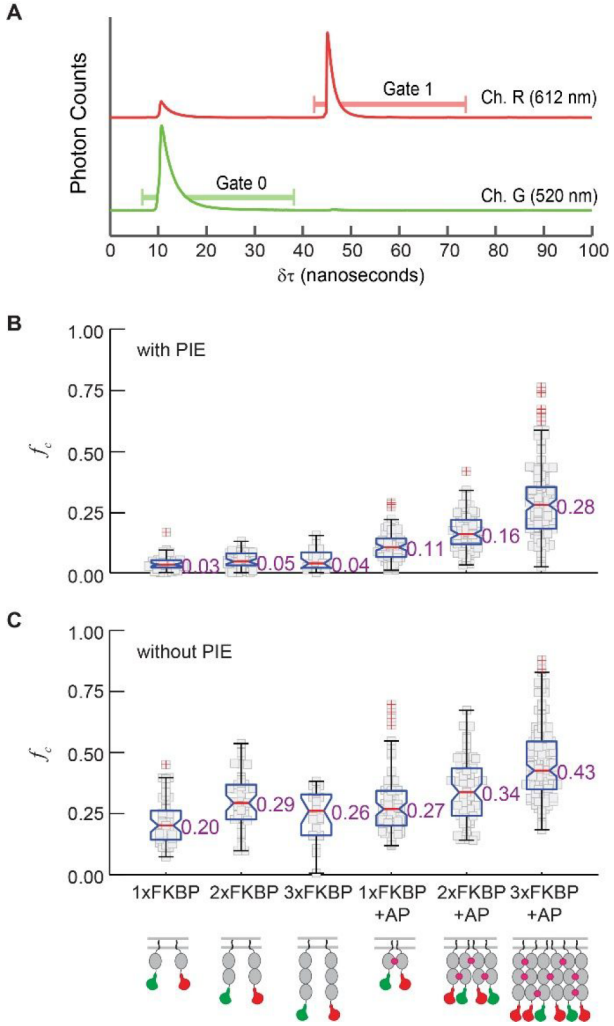

Figure 2. Demonstration of PIE-FCCS principle. (A) Example fluorescence lifetime histograms are shown with excitation at 488 and 561 nm. Photons detected in the red channel after 561 nm excitation are used to construct the intensity trace $I_{R,1}$, and photons detected in the green channel after 488 nm excitation are used to construct the intensity trace $I_{G,0}$. These two intensity traces are then used in the calculation of the ACF and CCF for PIE-FCCS experiments. (B) Cross-correlation (f_c) distributions are shown for single-cell measurements of several membrane protein controls described in the main text. Adapted with permission from ref 19. Copyright 2019 Elsevier. (C) The cross-correlation was recalculated from the same data but without applying the time gate.

R), resulting in four intensity traces: $I_{R,0}$, $I_{R,1}$, $I_{G,0}$, and $I_{G,1}$. For an experiment utilizing eGFP and mCherry as the fluorescent probes, the excitation sources can be blue (488 nm) and yellow-green (561 nm), and the emission channels will be green (~520 nm) and red (~612 nm). If the blue laser arrives in gate 0, then $I_{G,0}$ represents green emission after 488 nm excitation. The intensity trace $I_{R,1}$ is emission in the red channel after 561 nm excitation. These two signals ($I_{G,0}$ and $I_{R,1}$) are used to calculate the ACF and CCF in PIE-FCCS. The intensity trace $I_{G,1}$ is green emission upon 561 nm excitation, which is rare and is usually not significantly higher than the background detector noise. The signal in $I_{R,0}$ is red emission after 488 nm excitation, which results from direct excitation of mCherry, spectral bleed-through of eGFP, and energy transfer from eGFP to mCherry. These factors can significantly affect the CCF, and are excluded from the analysis.

Cross-correlation results for membrane protein controls are shown in Figure 2 with and without applying the time gate. The control constructs are a fusion of the FK506 binding protein (FKBP) to an N-terminal myristoylated peptide for membrane localization.[19] Each of the membrane constructs is monomeric, but undergoes dimerization or oligomerization after addition of a small molecule (AP20187) that cross-links the FKBP domains (Figure 2B).[19] In Figure 2C, the time-gate is removed from the analysis, which results in spectral cross-talk that increases the f_c distributions. For these data, the difference between the three monomer controls is now statistically significant, and the changes to each construct upon AP treatment are not consistent with the PIE-FCCS results. The results demonstrate that PIE is a simple and robust method for the removal of spectral cross-talk from FCCS measurements even in the heterogeneous environment of the plasma membrane.

Several experimental factors limit the observed degree of cross-correlation and the experimental f_c distribution.[20−22] FRET, for example, can reduce the observed degree of cross-correlation in FCCS experiments, but PIE can correct for this effect.[1,21] Chromatic aberrations cause a mismatch in the excitation volumes for two-color excitation. Fluorescent protein fusions provide specificity, but incomplete maturation and flickering can result in high dark state populations. Two-color labeling for measuring homo-oligomerization results in the statistical dilution of species with both red and green probes compared to species with only probes of one color. We have recently published a probabilistic model that accounts for the factors above, as well as the effect of dimerization affinity and the degree of oligomerization.[19] This model has allowed us to compare oligomer sizes based on the f_c distributions as will be described below.

CASE STUDIES OF FCCS IN MEMBRANE PROTEINS

Receptor Tyrosine Kinases

RTKs are single-pass transmembrane proteins that guide cellular processes like growth and differentiation. The canonical model of RTK activation was first described for EGFR by Yarden and Schlessinger in 1987.[23] For EGFR, ligand binding drives dimerization by inducing a conformational change of the extracellular domain, which is coupled to the formation of a catalytically active, asymmetric kinase domain dimer.[24,25] Many advanced fluorescence methods have been used to probe the oligomer state of EGFR before and after ligand binding,[2] including SMT, time-resolved anisotropy, photon counting and intensity distribution analysis, as well as an FCCS method using a single excitation source.[26] A key insight from the live-cell fluorescence studies was that the oligomerization state of EGFR is a dynamic equilibrium between monomers, dimers and multimers. This dynamic association requires methods that are sensitive to monomer and dimer populations in a live cell context. The exact distribution of monomers and oligomers is dependent on parameters like receptor density, cell type, and coreceptor interactions.

In 2013, PIE-FCCS was used to measure the degree of EGFR dimerization in model cell lines at normal physiological expression levels (Figure 3).[27] The concentration of labeled receptors was quantified for each cell with the ACFs and was

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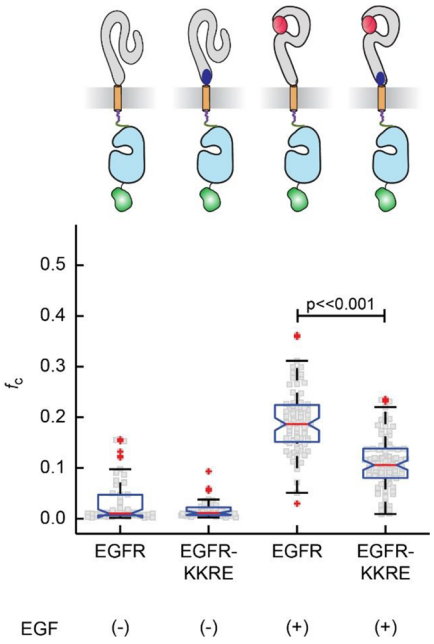

Figure 3. PIE-FCCS results for EGFR in live cells. Unstimulated, wild-type EGFR is monomeric in Cos-7 cells at expression levels around 10^2 and 10^3 receptors/μm^2. When stimulated with EGF ligand, the cross-correlation increases to a level consistent with multimerization. These ligand-bound multimers can be reduced to simple dimers by a point mutant in the extracellular domain. Reproduced with permission from ref 9. Copyright 2016 eLife.

found to vary between 10^2 and 10^3 molecules/μm^2. Diffusion coefficients were also determined from the ACF data. Dimerization was assessed from the amplitude of the cross-correlation function, f_c, for which the median value was 0.01 for unstimulated receptors. This result suggests that the ligand-free dimer population of EGFR is low under normal conditions but does not rule out a small fraction of dimers and multimers. PIE-FCCS data on a membrane anchored EGFR kinase domain showed that it was also monomeric under the same conditions, suggesting that the default conformation of the kinase domain at the membrane is to remain monomeric and inactive.[27] Molecular dynamics simulations indicated that this autoinhibition is stabilized by EGFR-lipid interactions.[28]

After ligand binding, EGFR undergoes a transition to a dimeric state that can further assemble into multimeric complexes.[29] Recently, a key interface that regulates the dimer/dimer interface was discovered in domain IV of the EGFR ectodomain.[9] PIE-FCCS was instrumental in resolving a reduction of EGFR oligomerization in live cells when that interface is mutated.[9] This ability to resolve changes in oligomer size is due to the sensitivity of FCCS to the statistics of two-color labeling.[19] The multimer distribution was also quantified with step-photobleaching experiments in oocytes and was linked to receptor activity. There are still several important details about the structure of the ligand-bound multimer that need to be resolved. For example, an alternative arrangement of the protomers was proposed in a subsequent paper using single-particle localization to measure the intermolecular distances within ligand-bound multimers in fixed cells.[30] High-resolution structure methods will likely be necessary to resolve these conflicts; however, the interfaces will need to be tested in situ with quantitative biophysical methods like FCCS.

To date, EGFR and ErbB2 are the only mammalian RTKs that have been investigated with live-cell FCCS methods. Several plant receptor kinases (RKs) have been studied with FCCS.[31,32] Like mammalian RTKs, plant RKs are also single-pass transmembrane proteins,[33] but can display wider substrate specificity that includes tyrosine and serine/threonine phosphorylation. Much less is known about the structure–function relationships in plant RKs; however, FCCS was applied specifically to measure their oligomeric state in live plant cell membranes. For two receptors, BRI1 and SERK1, a modest amount of cross-correlation was observed (15% compared to the linked dimer control), suggesting that the receptors are mostly monomeric with a small fraction of dimers.

G Protein-Coupled Receptors

The largest class of eukaryotic membrane proteins are seven-transmembrane helical proteins called G protein-coupled receptors (GPCRs). GPCRs are rich pharmaceutical targets because they sense a wide variety of chemical cues. When isolated, most GPCRs are functional as monomers, but there is an intriguing hypothesis that they can assemble into homo or heterodimeric complexes in the PM.[34] These quaternary interactions could have agonist or antagonist effects, but establishing the strength and stability of the interactions is experimentally challenging. Part of the challenge is that many of the GPCR dimers that had been identified previously have a relatively short lifetime (200–400 ms).[35] This suggests that the driving forces for dimerization are modest, and that environmental factors are likely to play a significant role. Consequently, GPCR dimerization must be investigated with quantitative methods that are sensitive to monomer/dimer distributions and can be performed in the live cell PM.

The first application of FCCS to study GPCR dimerization was on the corticotropin-releasing factor receptors (CRFRs).[36] CRFRs are class B GPCRs involved in the endocrine stress response and several behaviors including anxiety and depression.[37] Teichman et al. characterized the oligomerization state of two CRFRs using a combination of FCCS, FRET, and co-IP assays.[36] They found that CRF_1R, which has a cleavable signal peptide, is oligomeric in the PM of HEK 293 cells. Another CRFR, $CRF_{2A}R$, which has a noncleavable pseudo-signal peptide, was found to be monomeric. By swapping the N-terminal domains, they were able to alter the oligomerization potential: CRF_1R with the pseudosignal peptide became monomeric, while $CRF_{2A}R$ with the cleavable peptide showed modest oligomerization. Later, the same lab used FCCS to show that the CRF_1R oligomers are formed in the endoplasmic reticulum, prior to being trafficked to the PM.[38]

The oligomerization state of rhodopsin and human cone opsins has also been probed with PIE-FCCS.[39] Rhodopsin is a member of the class A GPCR family and has served as a prototype for GPCR structure and function studies. It is expressed in rod cells that contain stacks of invaginated PM disks in which rhodopsin is packed at high densities.[40] A low resolution cryo-EM structure revealed rhodopsin as a homodimer associated with a heterotrimeric G-protein.[41] Cryo-sectioning and tomography measurements also showed that rhodopsin assembled into highly ordered oligomeric structures in tissue.[42] Despite these observations there has been some controversy about the stability of these oligomeric structures. In 2014 we applied PIE-FCCS to resolve rhodopsin dimerization in a heterologous expression system (Figure 4).[39]

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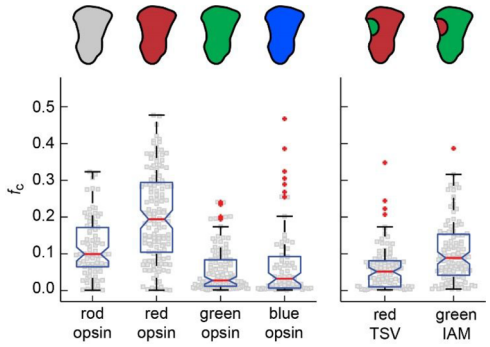

Figure 4. PIE FCCS data of rod and cone opsins. Single-cell cross-correlation values (f_c) are shown for each of the major photoreceptor pigments in humans. The rod opsin results are consistent with simple dimerization, while the red cone opsin results suggest that it can form multimers of various sizes. Green and blue cone opsins show low cross-correlation, similar to monomer control constructs. Triple point mutants, red-TSV and green-IAM, show a disruption of red cone opsin dimerization and induction of green cone opsin dimerization, respectively.[44]

The cross-correlation observed for apo-rhodopsin, or opsin, had a median value of 0.11, which was consistent with simple dimerization. The measurements were performed at concentrations ranging from 10^2 to 10^3 molecules/μm^2 and supported the hypothesis that rhodopsin can form oligomeric complexes in rod cells.

The dimerization potential of human cone opsins has also been studied with PIE-FCCS. The only prior investigation of oligomerization for cone opsins was reported in 2011, using confocal images as the primary characterization method.[43] To directly quantify dimerization, we conducted PIE-FCCS measurements on three human cone opsins (Figure 4).[44] We found that red cone opsin (OPN1LW) showed high cross-correlation consistent with strong dimerization. Green and blue cone opsins (OPN1MW and OPN1SW) showed f_c distributions consistent with a monomeric state. The difference in dimerization between red and green cone opsin was initially surprising because they share over 95% sequence homology, compared to 40−50% homology with the other human opsins. A swap mutant strategy was employed to replace amino acid residues in red and green cone opsins, leading to the identification of hot spot residues 230, 233, and 234 in TM5 (Figure 4). The red cone opsin swap mutant had a significant reduction in cross-correlation, and the green cone opsin swap mutant had an increase in the cross-correlation. This is the first set of GPCR mutations that can induce dimerization in an otherwise monomeric protein. Functionally, the TM5 amino acid residues have also been identified as key residues in the spectral sensitivity of red and green cone opsins. Amino acid residues 230 and 233 are responsible for approximately 4 nm spectral shift between red and green cone opsins. Together, these results suggest that the dimerization of red cone opsin could be allosterically modulating the spectral sensitivity and was part of the evolutionary drive for humans to adopt three color vision.

CONCLUSIONS AND OUTLOOK

Here, we reviewed PIE-FCCS and the application of FCCS to study membrane protein interactions in live cells. In the examples above, we attempted to highlight the key insights available from FCCS studies. First, FCCS is directly sensitive to the concentration of diffusing receptors in the membrane, making it possible to monitor single-cell expression levels and correlate dimerization with concentration. Second, FCCS provides direct information about the protein mobility, which is a secondary verification of the size of the diffusing species and their sensitivity to perturbations like ligand binding or point mutations. Third, cross-correlation is a rigorous indicator of stable interactions and can be used to measure associations even in cases where the probes may be poorly positioned for FRET. Finally, for PIE-FCCS the TCSPC data collection also enables fluorescence lifetime quantification, making it possible to simultaneously measure FRET and FCCS. This makes PIE-FCCS an information rich tool that allows for a quantitative characterization of the spatial organization of membrane proteins in situ. The combined features of PIE-FCCS make it robust even in the complex, heterogeneous environment of the plasma membrane.

Most membrane proteins that have been studied with FCCS fall into three major classes: RTKs, GPCRs, and cytokine/immune receptors. This early work has only scratched the surface of the protein diversity of the PM. Over 30% of the human genome encodes for membrane associated proteins, and the protein composition by weight of the PM is around 50%. These facts and many years of experiments illustrate that the PM is chemically complex, crowded, and heterogeneous. A major challenge in this field is determining the network of interactions that a given membrane protein experiences in the PM and how it regulates function at the protein, cell, and organism level. RTKs, for example, undergo a wide range of heteromeric interactions, but the quantitative details have only been investigated for a few proteins in a narrow set of cell types.[45] The GPCR superfamily contains hundreds of receptors, and an important frontier is deciphering hetero-dimerization between different receptors. A recent FCCS study investigated the interactions between the angiotensin type 2 receptor (AT2R) and MAS receptors.[46] Another important area is the role that dimerization plays in G protein binding. In one recent example, single-molecule step photobleaching and FCCS were used to show that the M2 muscarinic receptor is oligomeric in live cells and that its G protein, G_{i1}, is also oligomeric.[47,48]

Finally, quantitative biophysical methods like PIE-FCCS will be critical to establish the structure−function relationships of receptors that have received less attention. Cryo-EM and improved crystallization methods have begun to resolve atomic level details of membrane proteins at an astonishing rate. These new efforts can then be coupled with live cell biophysical assays like PIE-FCCS to determine how dimerization and other quaternary interactions regulate protein function.

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Notes

The authors declare no competing financial interest.

Biographies

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