Fluorescence-based Detection of Proteins and Their Interactions in Live cells

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Abstract

Recent advances in fluorescence-based microscopy techniques, such as single molecule fluorescence, FRET, fluorescence intensity fluctuations analysis, and super-resolution microscopy have expanded our ability to study proteins in greater detail within their native cellular environment and to investigate the roles that protein interactions play in biological functions, such as inter- and intra-cellular signaling and cargo transport. In this perspective, we provide an up-to-date overview of the current state of the art in fluorescence-based detection of proteins and their interactions in living cells, with an emphasis on recent developments that have facilitated the characterization of the spatial and temporal organization of proteins into oligomeric complexes in the presence and absence of natural and artificial ligands. Further advancements in this field will only deepen our understanding of the underlying mechanisms of biological processes and help develop new therapeutic targets.

1. INTRODUCTION

Fluorescence-based methods have become indispensable tools for monitoring protein expression, transport, folding, and localization within living cells.[1] Additionally, fluorescence-based techniques have been extensively used to study protein-protein and protein-ligand interactions, both of which play a critical role in cellular processes such as signal transduction and molecular transport.[2] Accurate detection of protein interactions is crucial for understanding cellular behavior and for developing new treatments for diseases associated with abnormal protein interactions. Recent technological and methodological advances have significantly benefitted biological studies using fluorescence. Such advances include (i)

development of high-sensitivity detectors (such as high efficiency photomultiplying tubes, electron-multiplying CCD cameras, and CMOS cameras) and performant excitation schemes with increased image acquisition speed, (ii) improved fluorophore labeling strategies, such as the use of brighter and/or more photostable fluorescent probes, which provide higher signal-to-noise ratios and enable more accurate quantification of oligomerization events, and (iii) advances in data acquisition and analysis techniques, such as the development of improved statistical methods for data interpretation and automated image analysis algorithms. These significant advancements have enabled researchers not only to detect the localization and motion of proteins with high resolution (including super-resolution), but also to probe and accurately quantify their interactions, in terms of the number of interacting partners, the stability of the supramolecular complexes they form, and even the geometry of those complexes. We will review herein the relevant fluorescence techniques used to image protein distributions, mobility, and interactions in living cells and highlight some of the recent studies that have contributed to advancing our knowledge of cellular and molecular biology and can lead to practical applications.

2. SINGLE MOLECULE STUDIES

2.1 Protein motion tracking

A powerful method for tracking protein motion in live cells or in vitro with exquisite resolution is based on determining the center of the point spread function of a microscope from fluorescence micrographs of individual molecules of interest tagged with one or more fluorophores. Refinements in this approach to achieve good signal to noise ratio led to the introduction of the technique of Fluorescence Imaging with One Nanometer Accuracy (FIONA)[3]. FIONA was used to successfully demonstrate the step size with extremely high precision of myosin motors as they translate down the length of actin filaments. It contributed not only to the understanding of the specific mechanisms involved in the function of such molecular motors but also implicitly established industry standards for pinpointing the center of the PSF spot with nm level resolution[3], which has been used in some super-resolution microscopy techniques (see section 5).

Recently, a modified version of the FIONA technique has been applied to investigate the trafficking of AMPARs during the early and maintenance phases of long-term potentiation (LTP) in living hippocampal cultured neurons[4]. Long-term potentiation (LTP) is a critical biological phenomenon which refers to the long-

lasting strengthening of synaptic connections between neurons that occurs as a result of repeated stimulation and is thought to be a critical cellular mechanism underlying learning and memory in the brain. While an increase in the number and conductance of postsynaptic AMPARs has been shown to mediate the expression of LTP, it is unclear how AMPARs are trafficked to the synapse during the early and maintenance phases of LTP. Jin et al. [4] conducted a study to investigate the molecular mechanism underlying the expression and maintenance of LTP in the central nervous system. They used single-molecule tracking with high localization resolution to quantitatively measure the numbers, percentages, and movements of synaptic AMPARs during both the early (0-10 min after induction of LTP) and maintenance phases (10- 20 min) of LTP in living hippocampal cultured neurons at the single-synapse and single-molecule level. They found that AMPARs are exocytosed into both synaptic and extrasynaptic membranes upon LTP induction but accumulate at synapses during the maintenance period without affecting their net flow from the extrasynaptic to synaptic membrane. This suggests that LTP maintenance is contributed largely by synaptic insertion of AMPARs rather than the surface diffusion of exocytosed AMPARs from extrasynaptic to synaptic regions.

Labeling the receptor of interest in living cells for single molecule protein tracking presents a persistent challenge, as it demands the selection of probes that can bind specifically and accurately to the target molecule without perturbing its normal function, while also being sufficiently bright and stable to enable detection and tracking at the single molecule level. Ren et al[5]have recently developed a small Affimer protein probe which can be inserted into living cancer cells to image an endogenous protein called estrogen receptor alpha (ERα). ERα mutations are implicated in 70% of breast cancers, making them of considerable interest to image various mutants of ERα in living cancer cell lines. The Affimer protein itself is bound to either Alexa647 or Cy3B fluorophores and can efficiently label ERα by traveling through temporary pores in the cell membrane created by the toxin streptolysin O. Ren et al showed that the Affimer protein preferentially labels the Y537S ER mutant but can also differentiate between other ER mutants as well. They used Cy3B conjugated Affimer to investigate the interaction between Y537S and its ligand, E2, and found that the diffusion of single Y537S decreases by 10× in the presence of E2. This reduction in the diffusion coefficient can be explained by the recruitment of cofactor proteins to Y537S driven by E2 binding, which results in the formation of larger protein complexes that cause slower diffusion. The measurement

of the shift of diffusion coefficient by single-particle tracking can also potentially be a way to validate the binding property of a new drug to Y537S. Overall, this study demonstrates the successful development of a small binding reagent that can specifically and efficiently bind to an intracellular native biomolecule, particularly those in the nucleus, while keeping the cell alive, allowing for tracking of the protein with nanometer precision.

2.2. Protein co-localization for detection of protein-protein interactions

Protein co-localization is a fluorescence-based method used to detect receptor-receptor interactions by observing and tracking the physical proximity of two or more proteins within a cell. By quantifying the level of co-localization of separate molecules (either different or same species), researchers can infer whether the proteins of interest interact with like receptors (homo-oligomerization) or other receptors (hetero-oligomerization). Many different imaging methods have been used in conjunction with protein co-localization, including wide-field fluorescence microscopy, confocal microscopy, two-photon microscopy, and super-resolution fluorescence microscopy. Detailed information regarding the equilibrium distribution percentages of monomers and higher-order oligomers has been recently achieved by combining Total Internal Reflection Microscopy (TIRFM)[6,7] with advanced methods for the analysis of the images thus obtained. TIRFM takes advantage of the phenomenon of total internal reflection to restrict the excitation light to a very narrow region near the surface of the slide on which the sample is mounted, allowing visualization of single proteins with high spatial resolution in the cell membrane, while reducing unwanted signals from proteins from the cytoplasm beneath. In many colocalization studies, different fluorescent tags are used to label the subunits of a protein complex with different colors, and microscopes are equipped with at least two detection arms that simultaneously detect different wavelength bands.

Technically, two or more fluorescently labeled molecules co-localize if their signals originate from within the same three-dimensional sample voxel (which is projected onto a two-dimensional image pixel), whose dimensions are limited by the diffraction limit (i.e., the limitation imposed by light diffraction on how small a light spot can be). If the fluorescent labels for different subunits of a protein complex colocalize and/or move together for times comparable to or longer than their separate diffusion times, it is likely that the subunits are part of the same protein complex. Recent advances in the protein-colocalization technique have enabled researchers to characterize the dynamic equilibrium between monomers, dimers, or higher-

order oligomers of the protein of interest.[8] To achieve this, a standard set of image analysis steps is applied to TIRF images taken from one or multiple channels corresponding to different spectral ranges. Fluorescent spots must first be identified in each such bandpass channel using a spot detection algorithm, such as a 2D Gaussian filter. Next, a frame-by-frame colocalization analysis algorithm is applied to the identified fluorescent spots to quantify the degree of colocalization between the different fluorescently tagged proteins. If multiple bandpass channels are simultaneously obtained (multi-color co-localization), this colocalization analysis could involve using the intensity correlation quotient (ICQ),[9] Manders' overlap coefficient,[10] or other similar methods to determine if two spots are “colocalized”[11] If only a single fluorescent label is utilized (single-color co-localization), histograms of the signal intensities from each of the fluorescent spots identified in the TIRF image can be generated and fitted by the sum of two Gaussian functions. Comparison of the relative amplitudes of the Gaussians to those obtained on monomeric controls provides information on whether a spot is monomer-like or consists of co-localized proteins. Regardless of which method is used to detect colocalized molecules, the next step involves tracking the proteins over time to determine if they exhibit co-diffusion for a certain number of frames, which is indicative of an interaction event. The fraction of co-localized proteins that are assigned to mobile trajectories are then used to determine relative homo- or hetero-oligomerization levels.

A recent application of the quantitative colocalization approach was demonstrated by Kasai et al[12], where they used single-color co-localization to determine the dissociation constant of β2 homo-dimers and estimate their lifetimes in the plasma membrane (PM). Histograms of the signal intensities from each of the fluorescent spots identified in the TIRF images were fitted by the sum of two Gaussian functions to provide the relative abundance of fluorescence spots that resembled monomer-like and dimer-like particles. They found that β2 adrenergic receptors (B2AR) form stable homodimers with a dissociation constant of 1.6 copies/μm^2 and an average lifetime of 83 ms, but that various inverse agonists reduced the dimers stability. Notably, the inverse agonists were also shown to reduce the B2AR’s basal constitutive activity, measured via intracellular cAMP levels. In addition to characterizing the equilibrium dynamics of B2AR, Kasai et al added a GFP molecule to various G protein subunits. They then used multi-color colocalization to assess the level of recruitment of the G proteins to B2AR monomers and dimers under different conditions. The results showed that trimeric G-proteins were recruited to both B2AR monomers and homodimers in the

constitutive state. Inverse agonists specifically blocked the G-protein recruitment to GPCR homodimers, but interestingly, did not affect the recruitment to monomers. Importantly, taken together, these results indicate that G-proteins recruited to transient GPCR homodimers are responsible for inducing their basic constitutive signals. These results suggest novel drug development strategies to enhance or suppress GPCR homodimer formation.

One consistent challenge experienced in protein co-localization studies is achieving selective and efficient labeling of target proteins with fluorophores of multiple colors. Belaton et al[13] recently developed an innovative technique for analyzing receptor interactions in live cells using single-molecule co-tracking of up to four colors. They applied their technique to quantifying the stoichiometry and dynamics of the interferon-γ (IFNγ) receptor signaling complex in the plasma membrane of living cells. IFNg is a homodimeric agonist of IFNγ and recruits a copy of two different IFNγ subunits, i.e., IFNGR1 and IFNGR2 at the cell surface. Previously, there was a lack of consensus regarding whether the IFNγ subunits were recruited by the IFNg agonist or preassembled into complexes in lipid nanodomains. To address this question, Belaton et al developed variants of CFP and eGFP which orthogonally recognize two different anti-GFP nanobodies, termed “enhancer” (EN) and “minimizer” (MI), antibodies, respectively. Furthermore, each of the two nanobodies was conjugated with one of two different fluorescent labels: EN with either ATTO643 (^{AT643}EN) or DY-642 (^{DY752}EN), while MI was conjugated with either ATTO 488 (^{AT488}MI) or ATTO Rho11 (^{Rho11}MI). To quantify heterodimerization between the IFNγ subunits, 4 color TIRF images with alternating laser excitations were acquired of cells coexpressing IFNGR1 (labeled with either ^{AT488}MI or ^{Rho11}MI) and IFNGR2 (labeled with either ^{AT643}EN or ^{DY752}EN) at the cell surface. Rigorous multi-color co-tracking analysis between the different spectral channels was used to detect complex formations. They performed frame-by-frame co-localization analysis to detect particles within 150 nm of one another across spectral channels. Interaction events were identified as molecules co-diffusing for at least ten images in a timeframe. The relative heterodimerization levels were determined based on the fraction of co-localized particles assigned to mobile trajectories. Notably, the studies showed ligand-induced homo- and heterodimerization of IFNγ receptor subunits, and not preformed receptor dimers or oligomers, which was previously thought to be the case.

Analysis of protein colocalization data has recently been improved with the introduction of Bayesian or machine-learning approaches.[14,15] For example, Huang et al[16] developed a deep-learning convolutional neural network (DLCNN) that automatically distinguished between receptor monomers and larger complexes based on images of fluorescent spots. The main idea behind a DLCNN is to identify patterns or features in an image by applying filters to the image, which are mathematically convoluted with the image to produce feature maps. These feature maps are then used as inputs to develop subsequent steps (or layers) in the analysis process, each of which performs a specific computation. A layer takes as input the output from the previous layer, applies a set of mathematical operations, e.g., matrix multiplications or convolutions, which recognize specific features (such as edges, corners, or textures), reduce the spatial dimensions of the feature maps, and, finally, categorize the image into one of several classes. Huang et al developed and trained their DLCNN model to automatically distinguish between a receptor monomer and a receptor oligomeric complex by analyzing the 2D spatial patterns of fluorescent spots produced by single or multiple quantum dots. The model was utilized to predict whether a fluorescent spot derived from a fluorescently labeled receptor of interest – the C-X-C motif chemokine receptor 4 (CXCR4) – represents a monomer or an oligomer. The DLCNN model was applied to investigate the dynamic process of CXCR4 in living cells during the early signaling stage. The findings indicate that CXCR4 forms an oligomeric complex following activation with the SDF-1 α agonist; interestingly, this complex was internalized more rapidly than the monomer via the clathrin-dependent internalization pathway.

2.3. Single-molecule photobleaching as a means to quantify protein oligomer size

Photobleaching step counting (PSC) is a method used in fluorescence microscopy to determine the number of fluorescent molecules present in an oligomeric complex.[17] In this method, a small region of the sample is repeatedly excited with light, and the fluorescence intensity is monitored over time. As the excitation light causes the fluorescence to diminish (photobleaching), the fluorescence intensity will decrease until no fluorescence can be detected. By counting the number of steps necessary for the fluorescence to drop below a certain threshold, the number of fluorescent molecules in the region can be estimated. While conceptually straightforward, a major hurdle in the PSC approach is the presence of noise due to various factors, such as background fluorescence, variable fluorophore emission, fluorophore blinking, and other photophysical effects exhibited by fluorescent molecules, whether subjected to single- or two-photon

excitation.[18] A second major hurdle in PSC is the manner in which steps are identified and counted[17,19]. Visual inspection is the primary method to analyze stepwise photobleaching intensity traces with some assistance from noise reduction via edge-preserving signal filtering. However, with an increasing number of fluorophores, the process can become unreliable and subjective.[20]

To address such issues, novel photobleaching step-counting methods using Bayesian statistics and machine learning have been developed, but they are demanding in terms of data quality and computationally costly.[21] Hummert et al[19] recently developed a robust and reliable algorithm, called quickPBSA, for analyzing photobleaching step traces. The underlying principle of the framework is to perform a preliminary step detection for each fluorescence intensity trace and then refine the results iteratively. The final refinement step makes use of a Bayesian posterior – a probability distribution that represents updated beliefs about an unknown quantity, given the evidence already observed. In Bayesian statistics, the posterior is calculated using Bayes' Theorem, which relates the prior distribution (the initial beliefs about the parameter) to the likelihood (the probability of the observed data given the parameter) and the evidence (the observed data).[22] Specifically, in the quickPBSA algorithm, the posterior which models the photobleaching step traces incorporates the possibility of simultaneous bleaching events and also a penalty for too many bleaching events, and therefore incorporates critical information into determining step size and locations along the trace. The study by Hummert et al validates the quickPBSA framework with experiments on DNA origami carrying a known number of fluorescent labels to demonstrate its robustness and reliability, and it illustrates its usefulness by determining the copy number of nucleoporin 107 (NUP107) in the nuclear pore complex (NPC).

The ability of PSC to quantify the number of molecules in a molecular complex can reveal important information, regarding not only the size of the complex but also the role played by oligomerization in biological function. Liu et al[23] used photobleaching step analysis to assess the importance of oligomerization of the platelet-activation factor receptor (PAFR), a GPCR involved in platelet aggregation and inflammatory responses, in the particular signaling pathway initiated by the receptor. The study reports the formation of PAFR dimers and oligomers in transfected cell lines. TIRF images of Halo-tagged PAFR covalently labeled with non-cell-permeant fluorophores (i.e., fluorophores that do not enter the cell) were obtained. Photobleaching steps from thousands of different spots on the cell membrane were collected for

multiple PAFR concentration ranges, and the relative abundances of the monomers, dimers, and higher-order oligomers determined from the distribution of bleaching steps showed a density-dependent di/oligomerization behavior. More interestingly, Liu et al were able to assess the relationship between the extent of this PAFR oligomerization and the signaling bias of the receptor. The phenomenon of "biased signaling" arises from the fact that distinct agonists acting on the same GPCR can engage different effectors that lead to different cellular responses. PAFR is known to couple to both Gq/11 and Gi/o G proteins and recruit β-arrestin1 to trigger a signaling event.[24] The authors identified two symmetric PAFR dimer interfaces involving different receptor transmembrane domains. Cross-linking the interfaces stabilized the PAFR dimers and oligomers and resulted in increased G protein signaling and decreased β-arrestin recruitment, which indicates that GPCRs may regulate signaling bias through oligomer formation.

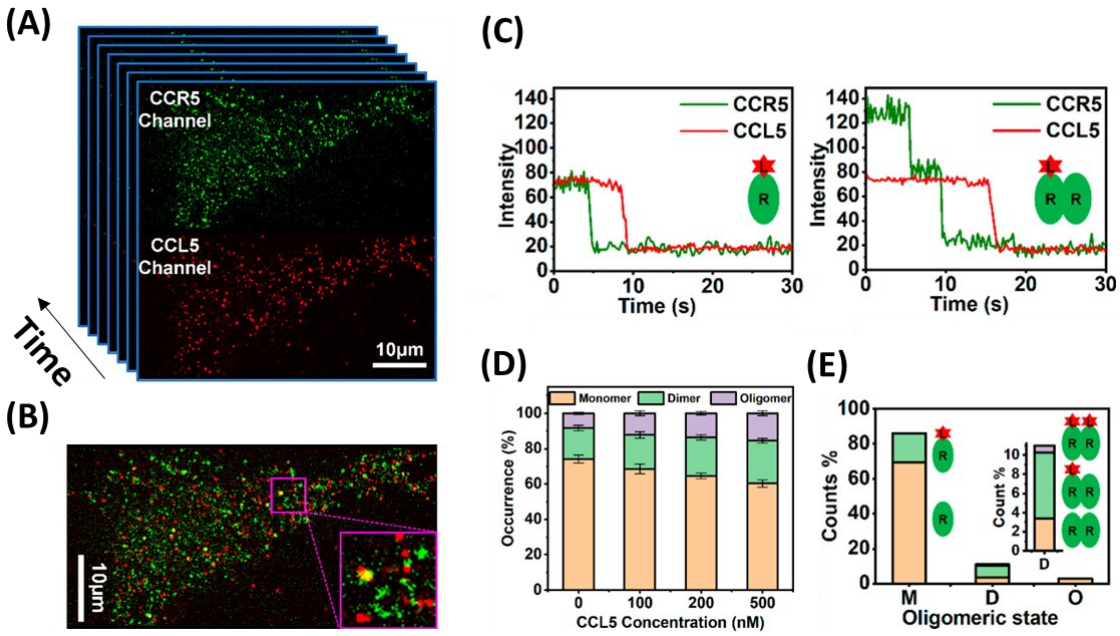

Figure 1. (A) Typical time series obtained from the dual-color TIRFM simultaneous tracking system; the top channel is for mNeonGreen-labeled chemokine receptors CCR5, and the bottom channel is Alexa Fluor-546 labeled CCL5 ligands**. (B)** CCR5 and CCL5 receptor–ligand binding complex photobleaching analysis. **(C)** Typical photobleaching steps of one CCR5 receptor binding with one CCL5 ligand and typical photobleaching steps of two CCR5 receptors binding with one CCL5 ligand (right panel). **(D)** Oligomerization of CCR5 on the cell membranes with the effects of CCL5 stimulation on the oligomeric status of CCR5 receptors. The oligomeric status of CCR5 receptors was analyzed according to the photobleaching steps of each spot. About 5000 spots from more than ten microscope fields and 15 cells were selected and analyzed at each expression level; the data represent the mean ± SE of three sets of parallel replicate samples. **(E)** Distribution of CCR5 monomers, dimers, and oligomers and their ligands binding analysis. (The inset is the enlarged version of column D, and the column O population is too small for ligand binding statistical representation). Adapted with permission from ref 25. Copyright 2022 American Chemical Society. ~~Figure was adapted with permission from [23].~~

PSC was also recently utilized to reveal a critical finding regarding the oligomerization properties of the chemokine receptor CCR5 in the presence of its agonist CCL5. Li et al[25] developed a dual-color simultaneous tracking system based on a TIRF microscope and stepwise photobleaching. The dual-color simultaneous tracking system (Fig. 1) allowed them to visualize and quantitatively assess the degree of oligomerization of CCR5 and to estimate the probability of binding between CCR5 and CCL5 for each oligomer state. CCR5 fused to the mNeonGreen fluorescent protein was expressed in Chinese ovary (CHO) cells and was found to form oligomers through TIRFM imaging combined with PSC. Low expression levels (0.11 particles/μm^2) showed a high propensity of CCR5 to be monomeric (99%), but, at higher expression, the dimer/oligomer populations increased while the monomer population decreased to 74%. Likewise, stimulation of CCR5 with the ligand CCL5 shifted the distribution of monomers and oligomers towards an even higher percentage of dimers and oligomers. Finally, CCL5 was directly labeled with Alexa Fluor 546 dye-via SNAP labeling procedure in order to investigate the binding ability of CCL5 for different oligomeric states. Dual color stepwise photobleaching results showed that each oligomeric state of CCR5 could bind with CCL5 but with different binding affinities. Interestingly binding of a CCL5 ligand to one protomer of a CCR5 dimer showed a two-fold higher binding affinity than CCL5 with monomers, but binding of ligand to the second protomer of a dimer complex with one ligand already bound showed a binding affinity half of that seen with a monomer.

The above-described effects that ligand binding has on the receptor oligomerization are important, as they add important details to the body of evidence available until recently (see next section). Discussion of earlier references on the topic of oligomerization studies using PSC can be found in the review article by Milstein et al [17].

3. FLUORESCENCE FLUCTUATION-BASED APPROACHES

3.1. Overview

In fluorescence fluctuation spectroscopy (FFS) studies, the random fluctuations in fluorescence intensity resulting from the movement of fluorescently labeled molecules into and out of the focal region of a light beam (Fig. 2) are collected and analyzed[26] to determine important properties of the molecules, including their diffusion coefficient and aggregation properties. Fluorescence fluctuation spectroscopy techniques

rely on analyzing the statistical moments of fluorescence intensity distributions (such as mean, variance, and skewness) to determine the molecular brightness of the fluorophore, i.e., the average detected signal of a single fluorescent molecule over a given time. The relationship between molecular brightness (average detected signal) and intensity stems from the fact that a larger complex of fluorescent proteins moving in or out of the region of interest will cause a larger fluctuation in fluorescence signal compared to a smaller one due to the number of fluorophores in the complex. Therefore, the molecular brightness of a complex of molecules scales linearly with the number of molecules within it. By monitoring the molecular brightness of a molecular complex under various conditions, moment-based FFS studies can monitor the dynamic behavior of individual fluorescently labeled proteins and provide insight into the oligomerization kinetics, stoichiometry, and stability of the resulting complexes. There are several types of fluctuation-based techniques, which fall roughly into two categories: space-based techniques, which quantify intensity fluctuations between pixels in an image, and time-based techniques, which monitor fluctuations in time for fixed position of the excitation beam.

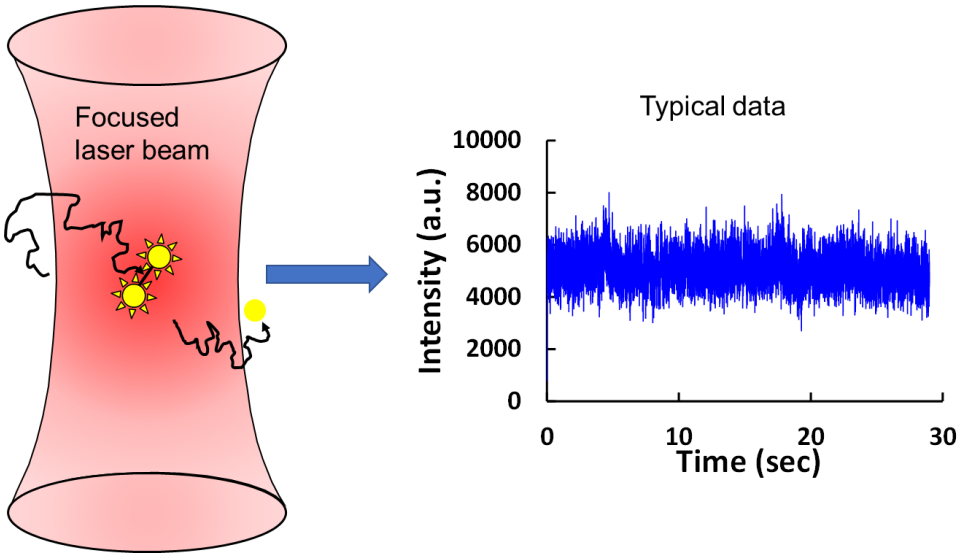

Figure 2. Principle of fluorescence fluctuation spectroscopy. (A) A focused laser beam illuminates the sample. When a single fluorescent particle enters the focal volume, a burst of fluorescent photons will be detected. When multiple fluorescent particles enter the volume, the intensity recorded from the burst of photons will be approximately twice as high as that recorded for the single fluorescent particle. By taking a large number of repeated intensity measurements in time (or space), the average size of the molecular complex can be ascertained by analyzing the corresponding width of the intensity distribution.

Number and Brightness (N&B) is a widely used fluorescence fluctuation method that uses a series of fluorescence images taken repeatedly on the same region of a cell over time to measure the average number of molecules and their oligomeric state in each pixel. The distribution of intensity values extracted from each pixel over the time course of the measurements along with the mean and variance of the intensity distribution are used to differentiate between different oligomeric states of the molecules at each pixel in the image via the calculation of the molecular brightness,[27] which scales with the size of the oligomer. This allows for the creation of pixel-by-pixel oligomerization maps of entire cells. N&B has been utilized to study aggregation numbers for several decades. However, traditionally, brightness values were determined for each pixel by dividing the variance by the average intensity of the individual pixel intensity distribution; this method of analysis leads to a large number of "outlier" pixels.

While N&B analyzes intensity fluctuations from the same pixel in a scan over time, a number of synonymous methods measure fluctuations in pixel intensities across space.[28-30] One such method, Two-Dimensional Fluorescence Intensity Fluctuation (2D FIF) spectrometry,[28] is a recently developed moment-based technique that provides information on the size and stability of protein oligomers as a function of receptor concentration. In 2D FIF, large regions of interest (ROIs) within the images are divided into smaller segments, and the intensity distribution of each segment is fit with a Gaussian function. The mean and variance of the fitted Gaussian are used to calculate a molecular brightness value and a concentration value for each segment. The brightness values are sorted by concentration into user-defined ranges, and a brightness distribution is compiled for each concentration range. The brightness spectrograms can be unmixed, based on knowledge of a monomeric brightness spectrogram, to obtain the relative contribution of the different-sized oligomeric species.

3.2. Oligomerization studies

Fukushima et al[31] implemented an empirical Bayes-maximum a posteriori (EB-MAP) estimation to N&B data, which utilizes the surrounding pixel information. The accuracy and precision of the EB-MAP approach were shown to be markedly better than the conventional method, by suppressing the number of outliers and thereby sharpening the 2D map of oligomerization for a given cell.

Two-Dimensional Fluorescence Intensity Fluctuation (2D FIF) spectrometry preserves information on fluctuations in oligomer size across the membrane and allows for the relative contribution of different-

sized oligomers to be determined. The method is robust even, when applied to images containing high-intensity puncta, which can artificially broaden the intensity distribution and cause artifacts. However, in some cases, the high-intensity puncta represent the objects of interest, such as lipid microdomains with different molecular compositions and supramolecular architecture than the surrounding membrane. Recently, Biener et al.[32] expanded the FIF approach to include analysis of protein-protein interactions within the small groups of pixels, or puncta, which present intensity values that are significantly higher than the typical intensity level of pixels in the surrounding region. The method is modified by (i) removing pixels associated with puncta from the analysis using a simple linear iterative clustering algorithm, and (ii) combining the pixel content of multiple (five) puncta of similar average intensity to obtain a single molecular brightness value. Brightness values are then calculated over multiple puncta clusters, organized into distributions, and analyzed similarly to the brightness distributions accumulated from larger segments in the membrane. The results show that the size of the oligomer entering puncta increases with receptor concentration and ligand treatment, and the density of puncta increases with ligand treatment duration, suggesting that they may represent pits that trap functional receptor oligomers and bud into endocytic vesicles.

Mercado et al[33] utilized FIF spectrometry to study the association of the protein EphA2 into dimers and high-order oligomers in response to different ligands as well as the correlation between the oligomer size of the EphA2 receptor and its signaling properties. EphA2 is part of the RTK family and its function is controlled through its oligomerization in the membrane. The study found that different ligands can induce different arrangements of the EphA2 extracellular region, leading to distinct receptor oligomerization states, which could be responsible for different signaling properties. A significant positive correlation was found between the efficacy of EphA2 Y588 phosphorylation in response to m-ephrinA1 and peptide ligands and the oligomer size, suggesting that the efficacy of EphA2 autophosphorylation may be modulated by agents that control oligomer size. The study also looked at the correlation between bias coefficients for EphA2 and the parameters of the EphA2 brightness distributions, but no correlation was found. Further investigations in different cell lines are needed to assess the validity of these conclusions. The authors also used the augmented spot-based FIF method described in the previous paragraph to separately analyze the pixels in the fluorescence images representing small, distinct puncta in order to study the oligomer size of EphA2 in

puncta. The results showed a large difference in the brightness distributions when comparing whole membranes to puncta, with the most common oligomer size being higher in the puncta for all ligands.

Ahmed et al.[34] utilized a combination of FRET and FIF spectrometry to test the hypothesis that ligand bias, or the ability of ligands to activate different signaling pathways through the same receptor, affects the stability of receptor tyrosine kinase (RTK) dimers. Fluorescence images of fluorescently labeled Trk-A were obtained in the presence of two different ligands, NGF and NT-3, each of which signals via a different pathway, leading to different biological outcomes. The authors used FIF to first assess the oligomeric size of Trk-A. The results showed that Trk-A, in the presence of NGF or NT-3, had molecular brightness distributions similar to the dimer control E-cadherin. The absence of higher-order oligomers suggests that neither NGF nor NT-3 induce the formation of oligomers larger than dimers. The results obtained from the FIF spectroscopy measurement were then used in conjunction with FRET data obtained from cells expressing the same fluorescently labeled Trk-A to probe the relationship between dimer interaction strength and bias toward a particular signaling pathway, which is discussed in more detail in section 4.2.

Discussion of earlier references on the topic of oligomerization studies using fluorescence fluctuation techniques, including a detailed description of the FIF spectrometry technique, can be found in the review article by Stoneman et al.[35]

3.3. Effect of ligand binding on receptor oligomerization

The research group led by Graeme Milligan at the University of Glasgow has recently used fluorescence fluctuation-based techniques to dig deeper into unresolved questions regarding GPCR oligomerization in living cells, namely, whether GPCR oligomerization is necessary for ligand binding and subsequent signaling and also whether experimentally observed GPCR oligomerization is a product of receptor expression levels being higher than in native cells. To this end, Ward et al.[36] used FIF spectrometry along with another spatial fluorescence fluctuation technique called spatial intensity distribution analysis (SPIDA)[29] to study the effect of ligand binding on the chemokine receptor CXCR4, which is of high interest due to its roles in cancer and viral infections. A large body of evidence indicated that CXCR4 forms at least dimers; however, conflicting results regarding the effect of ligand binding on this interaction and its importance in signaling have been reported. Previous studies using Bioluminescence Resonance Energy

Transfer (BRET), a process in which bioluminescent energy is transferred between two proteins in close proximity to one another, have shown receptor dimerization/oligomerization that is not affected by expression levels or the presence of ligands, while others indicate that ligand binding can alter the receptor's conformation. The results using FIF spectrometry indicated that CXCR4 did form dimers and higher-order oligomers, with the proportion of the latter increasing with expression levels. Two CXCR4 antagonists having distinct chemical structures, the small molecule antagonist isothiourea-1t (IT1t) and the clinically employed CXCR4 antagonist AMD3100, were used to study the effect of ligand binding on receptor oligomerization. Interestingly, Ward et al found that IT1t caused monomerization of the wildtype receptor, while AMD3100 had no effect on the oligomerization state. While it remains unclear why two different blockers of a receptor would have different effects on the ability of the receptors to interact, this study demonstrated both that CXCR4 is a good model system for investigating such a question and fluctuation-based techniques provide reliable ways for answering the question.

While experimental evidence exists from a large number of studies that many G protein-coupled receptors (GPCRs) can form dimeric and/or higher-order quaternary complexes, these observations were typically made in cell lines for which the expression levels of the receptors were higher than that seen in native cells or tissues. Advancements in fluorescence fluctuation methods have eventually brought us to the point where it is possible to identify the oligomeric state of transmembrane receptor proteins expressed at native concentration levels in native tissue. A major factor in the success of this effort is represented by the fact that intensity fluctuation-based methods only need one fluorophore-linked protein to be expressed in living cells when homo-oligomers are the target. Marsango et al[37] have successfully applied FIF spectrometry to neurons isolated from a line of transgenic "knock-in" mice in which the expression level and function of eGFP labeled M_1 muscarinic acetylcholine receptors were equivalent to the native M1 receptor in wild-type mice. The inherent ability of FIF to filter small clusters of high-intensity pixels from the surrounding plasma membrane proved vital in this study, as a substantial proportion of the receptors were present within intracellular punctate regions rather than being restricted to the plasma membrane. The results showed that a portion of the M1 receptors were present as monomers, but also that some dimers and even higher-order oligomeric complexes formed, even at the endogenous expression levels observed in these studies (20 to 60 molecules/μm^2). Interestingly, Marsango et al also found that both agonist and

antagonist ligands promoted the monomerization of the M1 receptor. These fundings corroborate previous findings involving muscarinic receptors and which were nevertheless conducted in non-native tissues and often at high expression levels.

4. FRET-BASED STUDIES OF HETERO AND HOMO-OLIGOMERIZATION

4.1. Overview

Since optical resolution is limited by the diffraction of a focused laser beam, it is not currently possible to directly resolve the distance between protomers within a molecular complex. Most of the methods discussed so far rely on indirect ways to infer whether two or more proteins physically interact with one another, without directly measuring the distance between them. Förster resonance energy transfer (FRET) – a process whereby the transition dipoles of excited optically fluorescent tags transfer their energy to unexcited molecules located within nanometers from it – allows direct detection of the distance between fluorescent tags based on the relationship between the efficiency of energy transfer (or FRET efficiency) and the distance between chromophores. The FRET efficiency is defined as the fraction of donor excitations transferred to acceptors via FRET, which ranges from 0 to 1 (sometimes given as a percentage of the total number of excitations transferred instead). It depends on the ratio (to the power of six) between a spectroscopic parameter called *Förster radius*, which incorporates several physical quantities, and the distance between the fluorescent tags.

Ensemble-based approaches to FRET based on plotting the average FRET efficiency versus, e.g., the donor concentration fraction,[38,39] can be used to probe the stability of homo- or hetero-interactions in membrane receptors, differentiate between monomers, dimers, or oligomers, and measure the free energies of association in the membrane,[40] provided that something is known about the oligomer size. In one of the more advanced embodiments of the ensemble-based FRET approach – the fully resolved spectral imaging FRET (FSI FRET)[38] –, the method uses two excitation wavelengths, pixel-level spectral resolution [41,42], and calibration curves of fluorescence versus known concentration of donor and acceptor solution standards for the determination of true protein concentrations. This data is then collected on

receptors of interest expressed over broad concentration ranges in the plasma membrane in order to fit dimer/oligomer binding models to the experimental data.

While ensemble resonance energy transfer-based techniques have been used effectively to quantify receptors dimers in cells, the time-averaged ensemble nature of the measurements masks any fluctuations between substates which may be occurring on the millisecond to second timescale. Conversely, single-molecule FRET is able to resolve and track individual pairs of donors and acceptors over time, observing an anti-correlated change in donor signal and acceptor signal. The primary ingredient in smFRET, in addition to the single-molecule sensitivity of the imaging system, is the use of FRET efficiency histograms, which allow for identification of different oligomeric configurations. Quantification of FRET at the single molecule level allows for the study of individual molecular interactions and conformations, leading to a more detailed understanding of biological processes at the molecular level.

A FRET-based method which relies on quantifying the FRET efficiency values measured between low concentrations of receptors, called FRET spectrometry,[35,43] has been designed specifically to determine detailed information about the size and shape of the quaternary structure of a protein complex and thereby reveal detailed information about the structure. FRET spectrometry involves collecting pixel-level values of the apparent FRET efficiency, E_{app} (i.e., the FRET efficiency of mixtures of oligomers of different sizes and possibly even monomers), assembling them into distributions (or histograms), and then extracting the most frequently occurring FRET efficiency values that appear as peaks in these distributions to form another distribution called a meta-histogram. The meta-histogram is then fitted with the FRET efficiency values predicted from various oligomer models, with the model producing the best fit taken as the quaternary structure of the oligomer. FRET spectrometry therefore combines features from both smFRET, which allows for detection and sorting of various oligomeric complexes according to their FRET efficiency values (i.e., size and geometry), and ensemble FRET approach, which allows for determination of proportions of oligomers of different sizes.

4.2. Probing protein homo- and hetero-oligomerization using ensemble FRET

A recent study by Sullivan et al utilized FSI-FRET to investigate the molecular details of the complex between E-cadherin and the epidermal growth factor receptor (EGFR) and its regulation by the EGF ligand and force.[44] E-cadherin complexes have been shown to activate EGFR signaling through an EGF-

dependent mechanism when mechanically perturbed in epithelial cells, a process which regulates cell contractility, stiffness, and the recruitment of actin and vinculin to perturbed cadherin adhesions.[45] There is evidence of crosstalk between E-cadherin and EGFR that regulates growth, but it is not yet clear how force and EGF alter the complexes and whether the two receptors directly associate. The stoichiometry of the complex is central to understanding the early steps in E-cadherin force transduction signaling. Results show that E-cadherin and EGFR form a heterotrimeric complex at the plasma membranes of live cells, which is disrupted by tension on homophilic E-cadherin bonds. EGF has the potential to disrupt the E-cadherin/EGFR heterotrimeric complex. It may do so by shifting the equilibrium towards more stable EGFR homodimers or by regulating the interaction between E-cadherin and EGFR. The study suggests that E-cadherin may inhibit EGFR by sequestering EGFR monomers and impairing homodimerization and signaling. However, EGF could override this inhibition and disrupt the complex, thereby potentiating EGFR signaling in epithelia. The mechanically activated release of EGFR suggests how junctional tension can sensitize epithelia to lower concentrations of EGF relative to unstressed tissues.

The high sensitivity and specificity of smFRET have made it a valuable tool in many areas of biophysics and molecular biology, including studies of protein-protein interactions, conformational changes in biomolecules, and molecular dynamics. However, due to significant experimental challenges, smFRET has primarily been applied to soluble proteins and application in mammalian cells has been limited. Recently, Asher et al. reported numerous advances in smFRET imaging that allowed for the study of receptor dimers in living cells, which includes self-labeling tags, bright self-healing fluorophores, and automated data-processing pipelines.[46] Their study showed that two GPCRs – the μ-opioid receptor and the secretin receptor – are monomeric at low surface densities in the membrane, while, at much higher densities, the secretin receptor forms long-lived complexes, demonstrating the density-dependent nature of receptor complex formation for at least some GPCRs. This corroborated results of a previous study of secretin receptors using a fluorescence intensity fluctuations-based approach.[28] They also used smFRET to track conformational changes in the ligand binding domains (LBDs) of metabotropic glutamate receptor 2 (mGluR2) dimers located in the plasma membrane of living cells. The distribution of FRET efficiency values in the unbound receptor case was centered at 0.46 and could be fit with a single Gaussian function, indicating either the absence of large-scale structural rearrangements or dynamics that greatly exceed the

imaging time scale. However, they found agonist-induced conformational changes between the mGluR2 LBDs resulted in a shift to a predominantly lower FRET state with a mean value of 0.29 in the presence of high concentrations of glutamate. Notably, analysis of the distribution of the FRET efficiencies measured in the presence of glutamate identified at least two FRET states within the population of oligomers.

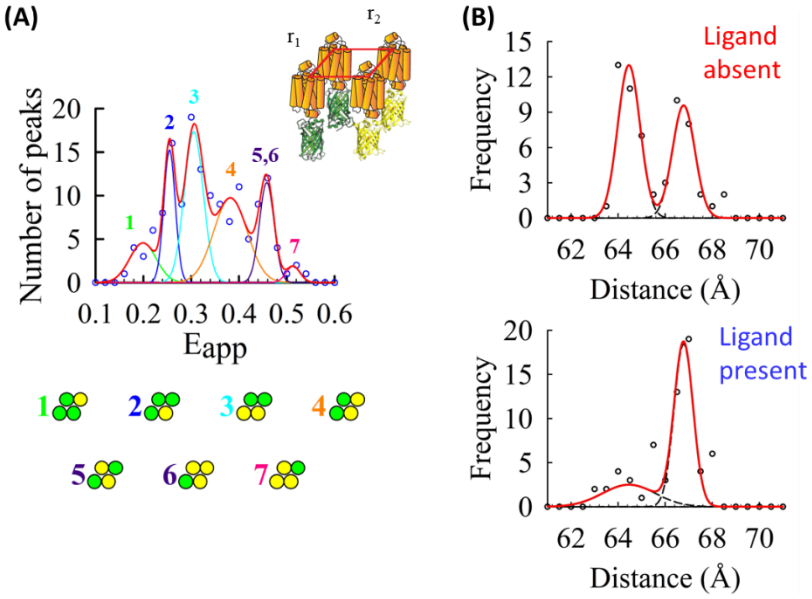

Figure 3. (A) Analysis of a metahistogram consisting of peaks of the histograms of apparent FRET efficiency (E_{app}; see section 4.1 for a definition) obtained from yeast (S. cerevisiae) cells coexpressing Ste2-GFP2 and Ste2-YFP, using a quaternary structure model. The metahistogram (empty blue circles) was fitted (solid red lines) to a parallelogram-shaped tetramer model using a reduced residual minimization algorithm. The model consists of seven Gaussian peaks (labeled 1-7) representing FRET-productive configurations of donors and acceptors within a tetramer. The side lengths (r_1 and r_2) and angle between sides of the parallelogram were adjusted during fitting to achieve the best fit. **(B)** Histograms of the frequencies of occurrence of one of the side length (r_2) of the tetramer obtained from fitting several metahistograms, before and after exposure to ligand. The multi-peaked distribution of the histograms indicates the existence of the Ste2 oligomer in multiple substates, which show a clear change in abundance after ligand addition. Adapted with permission from ref. 47. Copyright 2019 American Chemical Society. ~~Figure was adapted with permission from [45].~~

Recently, Paprocki et al[47] refined numerous steps in the FRET spectrometry approach such that even slight alterations (i.e., less than 1 Å) in the quaternary structure of membrane receptors could be detected. The improvement was achieved by implementing a noise-filtering algorithm, reducing the pixel area from which each FRET spectrogram was extracted, and narrowing the concentration ranges from which meta-histograms were generated. These refinements allowed the sampling of fluctuations in the histograms of FRET efficiencies, enabling the researchers to detect the presence of several quaternary structure conformations (or substates) in the sterile 2 alpha-factor pheromone receptor (Ste2) in living cells

(Fig. 3). They resolved four different quaternary structure conformations of Ste2, which were characterized by different distances between the protomers within an oligomer. The addition of a ligand shifted the relative abundance of the quaternary conformations, suggesting a possible relationship between quaternary structure substates and biological function. This research opens the possibility of studying the response of other membrane receptors to ligand binding in order to better understand the physiological relevance of oligomerization and exploit the potential therapeutic effects of biased signaling.

Discussion of earlier references on the topic of oligomerization studies using FRET, including a detailed description of the FRET spectrometry technique, can be found in a review article by Stoneman et al.[35]

4.3. Relationship between homo-oligomerization of receptors and binding of upstream (i.e., ligands) or downstream (effectors) signaling partners

Ligand bias, also known as functional selectivity, refers to the phenomenon where different agonists can promote differential receptor-induced signaling responses.[48] In other words, different agonists can activate the same receptor in a different manner, leading to distinct and selective activation of intracellular signaling pathways and differential regulation of cellular responses. The discovery of biased ligands highlights the importance of precise control of GPCR signaling for proper therapeutic action with fewer side effects. In other words, ligand bias can have important implications in drug design and therapeutic applications, as agonists with distinct signaling profiles can have different efficacy, safety, and side effect profiles. Many GPCRs can form dimers or oligomers and this may affect their G protein coupling selectivity.

FRET is beneficial for studying ligand bias because it provides a sensitive and specific method for measuring protein-ligand or protein-protein (whether homo- or hetero) interactions in real-time. Flöser et al[49] compared the agonist-induced binding of three GPCR-binding proteins, specifically, a G protein subunit (G_q, a G protein-coupled receptor kinase (GRK2), and a β-arrestin protein (arrestin3) to the muscarinic acetylcholine M3 receptors (M3R) using single-cell FRET imaging. The recruitment of Gq, GRK2, and arrestin3 to the M3R was studied directly (as opposed to analyzing downstream signaling) using FRET-based approaches to independently differentiate between their conformational requirements. The M3 receptor was labeled with the FRET donor (mTurquoise) and each of the three GPCR binding proteins was labeled with the acceptor (YFP). Flöser et al tested seven different agonists to determine if biased

recruitment of effector proteins could be detected at the M3R and if the bias in arrestin3 recruitment was caused by a bias in GRK2 recruitment. The results showed that ligand-induced biased recruitment was present at the M3R and that there was a difference between arrestin3 recruitment and the other two effector proteins, but no difference was found between GRK2 recruitment and G protein binding and activation, suggesting that similar receptor conformations are required for both.

FRET can be used not only to determine the bias of individual ligands but also to investigate any potential relationship between ligand bias and receptor oligomerization. Ahmed et al.[34] utilized a combination of FSI-FRET and FIF spectrometry to test the hypothesis that ligand bias affects the stability of receptor tyrosine kinase (RTK) dimers. Fluorescence images of fluorescently labeled Trk-A were obtained in the presence of two different ligands, NGF and NT-3, each of which signals via a different pathway, leading to different biological outcomes. The authors used FIF spectrometry to first assess the oligomeric size of Trk-A. The results showed that Trk-A, in the presence of NGF or NT-3, had molecular brightness distributions similar to the dimer control E-cadherin. The absence of higher-order oligomers suggests that neither NGF nor NT-3 induce the formation of oligomers larger than dimers. The results obtained from the FIF spectroscopy measurement were then used in conjunction with FSI-FRET data obtained from cells expressing the same fluorescently labeled Trk-A. Dimerization interaction strengths extracted from the FSI-FRET analysis show differences in the stability of NGF- and NT-3-bound Trk-A dimers, with NGF-bound Trk-A dimers being more stable than the NT-3-bound Trk-A dimers, supporting the hypothesis that ligand bias affects the stability of receptor tyrosine kinase (RTK) dimers. This could have implications for the design of Trk-A ligands with novel functionalities, based on how well they stabilize the Trk-A dimer, as well as many other RTK molecules.

FSI-FRET was also used to probe the relationship between ligand-biased signaling and oligomerization configuration in EphA2 receptors.[50] This study used EphA2-specific monomeric peptides to create dimeric ligands with different configurations to modulate EphA2 signaling responses. There were differences in signaling responses based on the peptide's configuration and they biased signaling through different mechanisms. Previous studies showed that agonist-induced EphA2 interactions cannot be explained by a simple dimer model, but instead suggest that the dimeric peptides induced larger EphA2 oligomers that utilize different interfaces. FSI-FRET studies were applied to two different mutants, each of which

possessed mutations to one of two interfaces known to be involved in EphA2 homo-oligomerization, one interface which has been named the “dimerization” interface and another named the “clustering” interface. The FSI-FRET studies showed that one of the dimeric peptides induced EphA2 oligomers utilized both the dimerization and clustering interfaces, while the second dimeric peptide induces EphA2 oligomers used only the dimerization interface. In other words, different dimeric peptides, which led to different signaling responses, induced EphA2 oligomers with a different configuration, i.e., that utilized different interfaces. This finding suggests that the large EphA2 oligomeric assemblies may be key to the ability of different dimeric ligands to differentially modulate EphA2 signaling.

5. DETECTION OF PROTEINS USING SUPER-RESOLUTION MICROSCOPY

5.1. Overview

Super-resolution microscopy has revolutionized biological research by enabling scientists to visualize cellular structures and processes with unprecedented detail at sub-diffraction resolution.[51,52] Over the last couple of decades, an array of super-resolution techniques has been proposed. Each of these techniques falls into one of three primary categories: stimulated emission depletion (STED), structured illumination microscopy (SIM), and single-molecule localization microscopy (SMLM).[53] Both STED and SIM make use of imaging systems employing advanced illumination setups to achieve sub-diffraction limited-resolution imaging of biological samples. In contrast, SMLM techniques rely on two principles to achieve sub-diffraction limited resolution: (i) The ability of individual fluorescent probes to switch between an active (or ‘ON’) state where they emit detectable fluorescent light, and inactive (or ‘OFF’) states in which they do not fluoresce, or their fluorescence appears undetectable on the camera. Different techniques rely on different mechanisms to achieve this switching between states, e.g. controlled laser irradiation,[54] chemical manipulation,[55] or rapid transient binding of the probes to the target molecules.[56] However, regardless of the technique, the end goal remains the same, to ensure that only a small number of molecules are active at any given time, resulting in spatially isolated fluorescent signals whose PSFs do not appreciably overlap. (ii) the location of a single fluorescing entity is found with much higher precision than what is possible using normal diffraction-limited microscopy by computing the centroids of the individual detected PSF. While each of the three categories has benefits and drawbacks, SMLM has a decided advantage in protein-interaction

studies due to the ability to achieve better resolutions (~20 nm on typical instruments) than STED (~50 nm) and SIM (~100 nm). Furthermore, reducing the effective fluorescence observation volume (and thereby improving the resolution) in STED setups entails a corresponding decrease in the total signal detected, whereas increasing the signal-to-noise ratio (by whatever means necessary) in SMLM setups only helps to increase the resolution.

The bevy of techniques encompassed by SMLM[17,53] each utilizes different means to realize the two key SMLM required steps listed above. The precise location of single molecules can be determined by fitting the emitted intensity distribution to the 2D spatial parameters of the point-spread function (PSF). The achievable resolution of SMLM techniques scales not with the wavelength of the excitation light, but as the inverse square root of the number of photons making up the PSF. In this regard, the SMLM techniques are directly related to the technique of Fluorescence Imaging with One Nanometer Accuracy (FIONA)[3] reviewed at the beginning of this article. Numerous methods exist for determining the center of a fluorescence spot, but one commonly used approach involves modeling the PSF with a two-dimensional Gaussian function. However, this simplistic model ignores the more intricate shapes of PSFs predicted by diffraction theory, leading to inaccuracies. More complex models based on optical theory can provide better PSF representations,[57] but often require additional parameters that must be determined beforehand. Consequently, it is advisable to calibrate PSF models before each experiment, using images of sub-diffraction fluorescent beads.[57] Ultimately, algorithms that rely on experimentally measured PSFs outperform those that utilize idealized PSF models.[58]

The original application of the FIONA technique relied on low expression levels (a single molecular motor) of the target of interest in order to achieve the requirement that the PSFs from two neighboring fluorophores do not overlap. For biological systems where the target of interest is more densely packed, alternative super-resolution methods must be utilized to avoid PSF overlap from neighboring fluorophores. To this end, multiple methods have been developed in which fluorophores can be turned “on” or “off” by selectively activating small subsets of fluorescent molecules with an additional excitation source, photoactivated localization microscopy (PALM)[54] or rely on stochastic fluctuations in the fluorescence of individual molecules, such as in stochastic optical reconstruction microscopy (STORM).[55] A more recent approach has been developed as an alternative strategy to those which rely on stochastic photo-activation

or deactivation of permanently bound fluorophores, such as PALM and STORM. This method, points accumulation for imaging in nanoscale topography (PAINT), is a process that uses dyes which can quickly and repeatedly bind and dissociate from the target to be imaged.[56] In PAINT, a fluorescent signal “turns on” and appears as a diffraction-limited spot emanating from the object of interest when a label binds to the target of interest, and the signal “turns off” when the label dissociates. The resulting "blinking" image captured by PAINT allows for the center of the fluorescence peak to be resolved with greater accuracy compared to conventional dye-based strategies, leading to a clearer and sharper image compared to conventional microscopy. The quality of the final image, which is created by overlapping a sequence of frames accumulated over time, depends on the diffusion coefficient and concentration gradient of the probes. DNA-PAINT [59] is a type of PAINT-based techniques that enables specific binding to a target of interest by using short complementary DNA sequences, one that is covalently attached to the protein of interest, and the other to a fluorophore that only fluoresces when the two DNA strands are bound.

SMLM has numerous applications in areas such as cell biology, neuroscience, and materials science, providing insight into cellular processes and interactions at the molecular level. However, despite the resolution offered by current super-SMLM techniques, until recently, protein-protein interactions and macromolecular complexes have still typically been analyzed with alternative methods. In the following paragraphs, we will discuss some of the breakthroughs over the last two years in applying SMLM techniques for the study of protein interactions.

5.2. Imaging proteins with super-resolution in living cells

Joseph et al[60] presented a new method for studying the oligomerization of the purinergic receptor Y2 (P2Y2), a rhodopsin-like GPCR, using SMLM in conjunction with the qPAINT technique (Fig. 4). qPAINT is a PAINT-based technique that relies on the repetitive binding between two complementary single-stranded DNAs, one labeled with a fluorescent dye (image strand) and the other to an antibody targeting the protein (docking strand). Emission only occurs from the fluorescent dye when the image strand is bound to the docking strand, and therefore the repetitive binding creates the blinking necessary to carry out SMLM measurements. Time-resolved TIRF images were obtained from cells expressing the P2Y2 bound to a docking DNA strands, and over the course of the collection of the time series, the DNA binding events form a cluster of single molecule localizations within the true position of the biological target. The frequency of

the single-molecule events is correlated with the number of labeled molecular targets, allowing for the quantification of P2Y2 molecular densities and spatial arrangements down to the single-molecule level. The authors applied this method to quantify the oligomerization status of P2Y2, a GPCR in the cancer cell line AsPC-1, and found that the receptor homo-oligomerizes and that its oligomerization state can be modulated by both agonists and antagonists, a finding that could inform efforts for its therapeutic targeting.

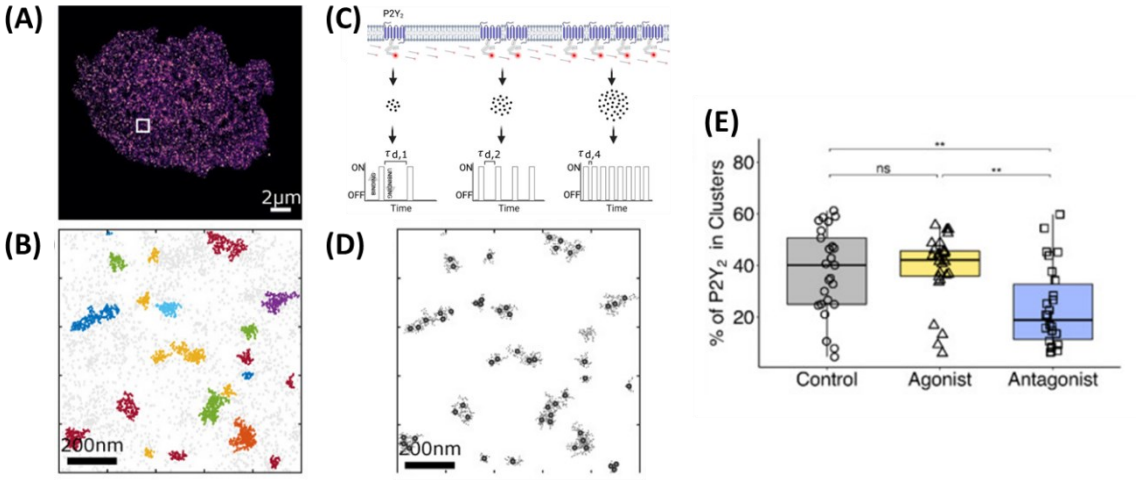

Figure 4. (A) Representative rendered DNA-PAINT images of P2Y2 proteins in AsPC-1 cells. White box indicates ROI for subsequent cluster analysis displayed in (B). **(B)** Output from cluster analysis algorithm, conducted using PALMsevier, applied to ROI indicated in (A). DNA-coupled antibody is localized several times, rendering a cluster of localizations (Colored dots) around the true position of the labelled proteins. Non-specific binding events are detected as non-clustered localizations (grey dots). **(C)** Schematic illustration of relationship between oligomer size and frequency of single molecule fluorescence events each docking strand is visited by an imaging strand at a frequency proportional to the concentration of the imaging strand and the association rate between imager and docking strand. The frequency of the imagers binding to their docking strand scales linearly with the number of docking strands, and this is the principle of the quantitative analysis known as qPAINT **(D)** Quantitative protein maps of the ROI indicated in (B) displaying the number of P2Y2 receptors (larger circles) in each cluster. **(E)** Percentage of P2Y2 receptors forming clusters of more than 5 proteins. Adapted with permission from ref. 60. Copyright 2021 MDPI. This figure was originally published under the terms of the CC BY 4.0 license (http://creativecommons.org/licenses/by/4.0).~~Figure was reproduced with permission from.~~

Fischer et al[61] described a new method called quantitative single-molecule colocalization (qSMCL) analysis, which combines quantitative single-protein imaging, genetically encoded control probes, cluster detection algorithms, and theoretical simulations, to determine absolute molecular quantities and the size of molecular complexes in living cells. QSMCL was applied to talin, kindlin, and integrin, to reveal the molecular densities and arrangement of these molecules, leading to the discovery of a ternary integrin-talin-kindlin complex in focal adhesions (FAs). To determine the exact number of protein molecules in a detected location, the authors calibrated the rate of blinking occurring due to binding and dissociation of the

imager strand to a protein of interest (either talin, kindlin, or integrin) with the rate of blinking seen from the image strand binding to a DNA origami structures with a defined number of docking strands placed near the protein expressing cells. The number of protein molecules in a given intensity cluster was determined by matching the observed rate of blinking from said cluster to one observed from a DNA origami; the number of docking sites on the DNA origami with the best match was taken to be the number of proteins in the given intensity cluster.

Harwardt et al used EXCHANGE-PAINT to study the presence of two RTKs, MET and EGFR, in two cancer cell lines.[62] Exchange-PAINT is a PAINT-based technique that takes advantage of the ability to wash out a DNA probe and image a second one; in this manner can quantify colocalization in two different species. They found that these receptors form heteromeric clusters in response to and activation. Furthermore, they observed that both MET and EGFR respond to their own as well as non-cognate ligands by slower diffusion. This is the first time that both static and dynamic evidence has been presented of the presence of heteromeric clusters of MET and EGFR in the cell membrane.

5.3. Combination of super-resolution with fluctuation-based or FRET methods

One area of great interest currently is the combination of super-resolution with other complementary techniques, such as fluctuation-based techniques, FRET approaches, or even two branches of super-resolution microscopy. By combining multiple techniques, it's possible to gain a more comprehensive understanding of the sub-resolution structure and dynamics of diffusing particles.

Sankaran et al [63] described a method for analyzing fluorescence intensity fluctuations at each pixel in a sequence of TIRF images to determine the diffusion maps and sub-resolution organization of diffusing particles using a combination of fluorescence fluctuation and super-resolution techniques. The method combines the techniques of Imaging Fluorescence Correlation Spectroscopy (Imaging FCS), N&B analysis (described above), and super-resolution radial fluctuations (SRRF). SRRF is a computational algorithm that analyses a sequence of images obtained from a standard TIRF microscope. SRRF works by analyzing fluctuations in radial symmetry of the image frames, based on the assumption that the PSF of fluorescent molecules have higher radial symmetry, or “radiality”, than the background. Radiality is measured for each subpixel in the image by taking a ring of neighboring subpixels and measuring the convergence of intensity gradients passing through these. SRRF then calculates the radiality on a sub-pixel basis for each frame in

the dataset. The combination of radial symmetry analysis with temporal analysis leads to further denoising and improved resolution. The authors demonstrated the utility of this method by applying it to monitor the super-resolved structure and dynamics of two different biomolecules, Lifeact (a small peptide that can bind to and label the actin cytoskeleton in living cells) and EGFR, in real time. Simultaneous determination, using two different emission wavelength channels, of the super-resolved structure of the cell cytoskeleton and the millisecond molecular dynamics of EGFR from the exact same pixels, enables the correlation between molecular structure and dynamics. N&B analysis of EGFR-mApple showed an intermediate brightness between monomeric and dimeric control brightnesses, indicating that it contains a mixture of EGFR monomers and dimers, with ~60% of the molecules participating in dimers. The diffusion coefficient of EGFR (D_E) was found to be 0.23 ± 0.22 $\mu m^2/s$. When the cognate ligand EGF (epidermal growth factor) was introduced, the value of D_E was reduced 3.8-fold, EGFR clusters appeared, and N&B analysis showed the formation of at least trimers. The correlation between SRRF (a measure of the super-resolved structure of the cytoskeleton) and brightness or diffusion coefficients was found to be close to zero, suggesting that actin does not have a direct effect on EGFR diffusion or oligomerization. However, an indirect effect on the diffusion coefficient, but not on the molecular brightness, was detected after disruption of the cytoskeleton was detected, indicating that actin indirectly affects EGFR diffusion through changes in cell membrane organization but does not increase oligomerization.

Moller et al[64] have combined multiple types of SMLM (i.e., protein tracking, co-localization, and dSTORM) with intensity fluctuation analysis to investigate the membrane dynamics and spatiotemporal organization of μOR. Simultaneous application of protein tracking to μOR and dSTORM to image actin fibers led to the discovery that μORs are subject to cell-surface compartmentalization, caused by diffusion barriers created by the actin fibers, and exhibit different types of diffusion behavior. Activation by agonists DAMGO and morphine caused a transient increase in the diffusion speed of the receptors, allowing them to cross these barriers more frequently. The study also found that μORs are predominantly monomeric, with dimer formation increasing in response to DAMGO, but not other agonists. Interestingly, the level of dimerization was similar to that measured for a mutant version of μOR which is constitutively active. This finding led the authors to suggest that phosphorylation-dependent binding of β-arrestins may be involved in μOR dimer formation, which would be intricately linked to agonist bias.

In all super-resolution methods discussed in this section, an increase in localization precision is achieved by collecting more photons, which is ultimately limited by the nature of the fluorescent tags as well as the image acquisition time. A recently developed technique,[65] which combines features from both STED and SMLM, achieves single-nanometer resolution by localizing single fluorophores according to the lowest emission fluxes that arise from a local minimum in excitation. This technique, called MINFLUX, uses a doughnut-shaped excitation beam to determine the position of a single fluorophore by moving it sequentially to four different positions. When the doughnut center is precisely aligned with the fluorophore position, no photons are emitted. The position of the fluorophore can be determined with high precision by analyzing the fluorescence photon counts from all four probing positions. In this manner, MINFLUX can achieve high localization precision, even with relatively dim fluorescent proteins, and >100-fold faster single-molecule tracking than competing methods. These new techniques have the potential to set a new standard for molecular-resolution SMLM with further advances in 3D, multicolor, and live-cell imaging.

Biographies

Michael Stoneman received his Bachelor's degree in Physics from the University of Wisconsin-Stevens Point in 2004 and his PhD from the University of Wisconsin-Milwaukee in 2010. Having first worked as a research scientist for a technology startup, he returned to academia and is currently an Associate Scientist at the University of Wisconsin-Milwaukee. Dr. Stoneman's current research is primarily focused on the development of optical techniques and their applications to detection and characterization of membrane protein complexes in living cells.

Valerică Raicu received his Physics Diploma (1991) and PhD (1998) from the University of Bucharest in Romania. He worked as a Research Scientist for the Institute of Physical Chemistry of the Romanian Academy of Sciences, and the Research & Design Institute for Rare and Radioactive Metals, and later as a Research Instructor at the Kochi Medical School, Department of Physiology, in Japan. In 2002, he obtained a Postdoctoral Fellowship at the Departments of Chemistry and Physics and the Charles H. Best Institute of Biomedical Research, University of Toronto. Since 2004, Dr. Raicu has been with the Department of Physics and the Department of Biological Sciences at the University of Wisconsin-Milwaukee, where he is currently a professor. Professor Raicu's main research interests include development of fluorescence imaging, FRET, and nonlinear optical micro-spectroscopy technology and its

use in studying protein quaternary structure in living cells, as well as quantum effects in fluorescent molecules.

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References

(1) Hickey, S.M.; Ung, B.; Bader, C.*; et al.* Fluorescence Microscopy-An Outline of Hardware, Biological Handling, and Fluorophore Considerations. *Cells* **2022,** *11*.

(2) Lu, H.Y.; Zhou, Q.D.; He, J.; Jiang, Z.L.; Peng, C.; Tong, R.S.; Shi, J.Y. Recent advances in the development of protein-protein interactions modulators: mechanisms and clinical trials. *Signa.l Transduct. Target. Ther.* **2020,** *5*.

(3) Kural, C.; Balci, H.; Selvin, P.R. Molecular motors one at a time: FIONA to the rescue. *J. Phys. Condens. Mat.* **2005,** *17*, S3979-S3995.

(4) Jin, C.; Jang, S.-S.; Ge, P.; Chung, H.J.; Selvin, P. Single molecule tracking of AMPA receptors shows the role of synaptic insertion during maintenance of chemical LTP. *bioRxiv* **2021,** *2021-02-04*, DOI: 10.1101/2021.1102.1104.429665.

(5) Ren, P.; Tiede, C.; Fanning, S.W.*; et al.* Labeling of a mutant estrogen receptor with an Affimer in a breast cancer cell line. *Biophys. J.* **2022,** *121*, 3651-3662.

(6) Axelrod, D., Optical configurations and setup, in Total Internal Reflection Fluorescence (TIRF) and Evanescence Microscopies. 2022, IOP Publishing. p. 5-1-5-32.

(7) Martin-Fernandez, M.L.; Tynan, C.J.; Webb, S.E.D. A 'pocket guide' to total internal reflection fluorescence. *J. Microsc.* **2013,** *252*, 16-22.

(8) Kasai, R.S.; Suzuki, K.G.N.; Prossnitz, E.R.; Koyama-Honda, I.; Nakada, C.; Fujiwara, T.K.; Kusumi, A. Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J. Cell Biol.* **2011,** *192*, 463-480.

(9) Li, Q.; Lau, A.; Morris, T.J.; Guo, L.; Fordyce, C.B.; Stanley, E.F. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J. Neurosci.* **2004,** *24*, 4070-4081.

(10) Manders, E.M.M.; Verbeek, F.J.; Aten, J.A. Measurement of Colocalization of Objects in Dual-Color Confocal Images. *J. Microsc.* **1993,** *169*, 375-382.

(11) Dunn, K.W.; Kamocka, M.M.; McDonald, J.H. A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol.* **2011,** *300*, C723-C742.

(12) Kasai, R.S.; Fujiwara, T.K.; Kusumi, A. Metastable GPCR dimers trigger the basal signal by recruiting G-proteins. *bioRxiv* **2020,** *2020-02-10*, DOI: 10.1101/2020.1102.1110.929588.

(13) Sotolongo Bellon, J.; Birkholz, O.; Richter, C.P.*; et al.* Four-color single-molecule imaging with engineered tags resolves the molecular architecture of signaling complexes in the plasma membrane. *Cell Reports Methods* **2022,** *2*, 100165.

(14) Ordabayev, Y.A.; Friedman, L.J.; Gelles, J.; Theobald, D.L. Bayesian machine learning analysis of single-molecule fluorescence colocalization images. *Elife* **2022,** *11*.

(15) Liu, X.L.; Jiang, Y.F.; Cui, Y.T.; Yuan, J.H.; Fang, X.H. Deep learning in single-molecule imaging and analysis: recent advances and prospects. *Chem. Sci.* **2022,** *13*, 11964-11980.

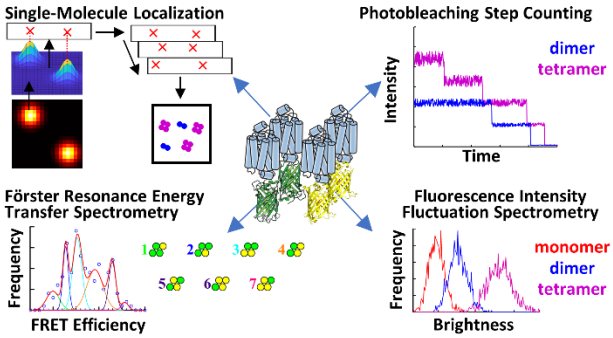
(16) Wang, Q.; He, H.; Zhang, Q.*; et al.* Deep-Learning-Assisted Single-Molecule Tracking on a Live Cell Membrane. *Anal. Chem.* **2021,** *93*, 8810-8816.

(17) Milstein, J.N.; Nino, D.F.; Zhou, X.H.; Gradinaru, C.C. Single-molecule counting applied to the study of GPCR oligomerization. *Biophys. J.* **2022,** *121*, 3175-3187.

(18) Adhikari, D.P.; Biener, G.; Stoneman, M.R.; Badu, D.N.; Paprocki, J.D.; Eis, A.; Park, P.S.H.; Popa, I.; Raicu, V. Comparative photophysical properties of some widely used fluorescent proteins under two-photon excitation conditions. *Spectrochim. Acta A Mol.* **2021,** *262*, 120133.
(19) Hummert, J.; Yserentant, K.; Fink, T.; Euchner, J.; Ho, Y.X.; Tashev, S.A.; Herten, D.P. Photobleaching step analysis for robust determination of protein complex stoichiometries. *Mol. Biol. Cell* **2021,** *32*.
(20) Garry, J.; Li, Y.C.; Shew, B.; Gradinaru, C.C.; Rutenberg, A.D. Bayesian counting of photobleaching steps with physical priors. *J. Chem. Phys.* **2020,** *152*.
(21) Madarshahian, R.; Caicedo, J.M. Reducing MCMC Computational Cost with a Two Layered Bayesian Approach. *Model Validation and Uncertainty Quantification, Vol 3* **2015,** 291-297.
(22) Rouder, J.N.; Morey, R.D. Teaching Bayes' Theorem: Strength of Evidence as Predictive Accuracy. *American Statistician* **2019,** *73*, 186-190.
(23) Liu, J.; Tang, H.; Xu, C.*; et al.* Biased signaling due to oligomerization of the G protein-coupled platelet-activating factor receptor. *Nat. Commun.* **2022,** *13*, 6365.
(24) Chen, J.; Lan, T.; Zhang, W.M.; Dong, L.J.; Kang, N.; Zhang, S.M.; Fu, M.; Liu, B.; Liu, K.T.; Zhan, Q.M. Feed-Forward Reciprocal Activation of PAFR and STAT3 Regulates Epithelial-Mesenchymal Transition in Non-Small Cell Lung Cancer. *Cancer Res.* **2015,** *75*, 4198-4210.
(25) Li, J.Q.; Ding, Y.Z.; Liu, H.H.; He, H.; Yu, D.Y.; Wang, X.Q.; Wang, X.J.; Yu, X.X.; Ge, B.S.; Huang, F. Oligomerization-Enhanced Receptor-Ligand Binding Revealed by Dual-Color Simultaneous Tracking on Living Cell Membranes. *J. Phys. Chem. Lett.* **2021,** *12*, 8164-8169.
(26) Weidemann, T.; Mucksch, J.; Schwille, P. Fluorescence fluctuation microscopy: a diversified arsenal of methods to investigate molecular dynamics inside cells. *Curr. Opin. Struct. Biol.* **2014,** *28*, 69-76.
(27) Paul, M.D.; Rainwater, R.; Zuo, Y.; Gu, L.; Hristova, K. Probing Membrane Protein Association Using Concentration-Dependent Number and Brightness. *Angewandte Chemie-International Edition* **2021,** *60*, 6503-6508.
(28) Stoneman, M.R.; Biener, G.; Ward, R.J.; Pediani, J.D.; Badu, D.; Eis, A.; Popa, I.; Milligan, G.; Raicu, V. A general method to quantify ligand-driven oligomerization from fluorescence-based images. *Nat. Methods* **2019,** *16*, 493-496.
(29) Godin, A.G.; Rappaz, B.; Potvin-Trottier, L.; Kennedy, T.E.; De Koninck, Y.; Wiseman, P.W. Spatial Intensity Distribution Analysis Reveals Abnormal Oligomerization of Proteins in Single Cells. *Biophys. J.* **2015,** *109*, 710-721.
(30) Isbilir, A.; Serfling, R.; Moller, J.*; et al.* Determination of G-protein-coupled receptor oligomerization by molecular brightness analyses in single cells. *Nat. Protoc.* **2021,** *16*, 1419-+.
(31) Fukushima, R.; Yamamoto, J.; Kinjo, M. Empirical Bayes method using surrounding pixel information for number and brightness analysis. *Biophys. J.* **2021,** *120*, 2156-2171.
(32) Biener, G.; Stoneman, M.R.; Raicu, V. Fluorescence intensity fluctuation analysis of receptor oligomerization in membrane domains. *Biophys. J.* **2021,** *120*, 3028-3039.
(33) Zapata-Mercado, E.; Biener, G.; McKenzie, D.M.; Wimley, W.C.; Pasquale, E.B.; Raicu, V.; Hristova, K. The efficacy of receptor tyrosine kinase EphA2 autophosphorylation increases with EphA2 oligomer size. *J. Biol. Chem.* **2022,** *298*, 102370.
(34) Ahmed, F.; Zapata-Mercado, E.; Rahman, S.; Hristova, K. The Biased Ligands NGF and NT-3 Differentially Stabilize Trk-A Dimers. *Biophys. J.* **2021,** *120*, 55-63.
(35) Stoneman, M.R.; Raicu, N.; Biener, G.; Raicu, V. Fluorescence-based Methods for the Study of Protein-Protein Interactions Modulated by Ligand Binding. *Curr. Pharm. Des.* **2020,** *26*, 5668-5683.
(36) Ward, R.J.; Pediani, J.D.; Marsango, S.; Jolly, R.; Stoneman, M.R.; Biener, G.; Handel, T.M.; Raicu, V.; Milligan, G. Chemokine receptor CXCR4 oligomerization is disrupted selectively by the antagonist ligand IT1t. *J. Biol. Chem.* **2021,** *296*.
(37) Marsango, S.; Jenkins, L.; Pediani, J.D.*; et al.* The M-1 muscarinic receptor is present in situ as a ligand-regulated mixture of monomers and oligomeric complexes. *Proc. Natl. Acad. Sci. U.S.A.* **2022,** *119*.
(38) King, C.; Stoneman, M.; Raicu, V.; Hristova, K. Fully quantified spectral imaging reveals in vivo membrane protein interactions. *Int. Biol.* **2016,** *8*, 216-229.
(39) Raicu, V.; Jansma, D.B.; Miller, R.J.; Friesen, J.D. Protein interaction quantified in vivo by spectrally resolved fluorescence resonance energy transfer. *Biochem. J.* **2005,** *385*, 265-277.
(40) Paul, M.D.; Grubb, H.N.; Hristova, K. Quantifying the strength of heterointeractions among receptor tyrosine kinases from different subfamilies: Implications for cell signaling. *J. Biol. Chem.* **2020,** *295*, 9917-9933.

(41) Raicu, V.; Stoneman, M.R.; Fung, R.; Melnichuk, M.; Jansma, D.B.; Pisterzi, L.F.; Rath, S.; Fox, M.; Wells, J.W.; Saldin, D.K. Determination of supramolecular structure and spatial distribution of protein complexes in living cells. *Nat. Photon.* **2009,** *3*, 107-113.
(42) Biener, G.; Stoneman, M.R.; Acbas, G.; Holz, J.D.; Orlova, M.; Komarova, L.; Kuchin, S.; Raicu, V. Development and Experimental Testing of an Optical Micro-Spectroscopic Technique Incorporating True Line-Scan Excitation. *Int. J. Mol. Sci.* **2014,** *15*, 261-276.
(43) Raicu, V.; Singh, D.R. FRET Spectrometry: A New Tool for the Determination of Protein Quaternary Structure in Living Cells. *Biophys. J.* **2013,** *105*, 1937-1945.
(44) Sullivan, B.; Light, T.; Vu, V.; Kapustka, A.; Hristova, K.; Leckband, D. Mechanical disruption of E-cadherin complexes with epidermal growth factor receptor actuates growth factor-dependent signaling. *Proc. Natl. Acad. Sci. U.S.A.* **2022,** *119*.
(45) Muhamed, I.; Wu, J.; Sehgal, P.; Kong, X.; Tajik, A.; Wang, N.; Leckband, D.E. E-cadherin-mediated force transduction signals regulate global cell mechanics. *J. Cell Sci.* **2016,** *129*, 1843-1854.
(46) Asher, W.B.; Geggier, P.; Holsey, M.D.*; et al.* Single-molecule FRET imaging of GPCR dimers in living cells. *Nat. Methods* **2021,** *18*, 397-405.
(47) Paprocki, J.D.; Biener, G.; Stoneman, M.R.; Raicu, V. In-Cell Detection of Conformational Substates of a G Protein-Coupled Receptor Quaternary Structure: Modulation of Substate Probability by Cognate Ligand Binding. *J. Phys. Chem. B* **2020,** *124*, 10062-10076.
(48) Slosky, L.M.; Caron, M.G.; Barak, L.S. Biased Allosteric Modulators: New Frontiers in GPCR Drug Discovery. *Trends Pharmacol. Sci.* **2021,** *42*, 283-299.
(49) Floser, A.; Becker, K.; Kostenis, E.; Konig, G.; Krasel, C.; Kolb, P.; Bunemann, M. Disentangling bias between G(q), GRK2, and arrestin3 recruitment to the M(3) muscarinic acetylcholine receptor. *Elife* **2021,** *10*.
(50) Gomez-Soler, M.; Gehring, M.P.; Lechtenberg, B.C.; Zapata-Mercado, E.; Ruelos, A.; Matsumoto, M.W.; Hristova, K.; Pasquale, E.B. Ligands with different dimeric configurations potently activate the EphA2 receptor and reveal its potential for biased signaling. *iScience* **2022,** *25*, 103870.
(51) Valli, J.; Garcia-Burgos, A.; Rooney, L.M.; Oliveira, B.V.D.E.; Duncan, R.R.; Rickman, C. Seeing beyond the limit: A guide to choosing the right super-resolution microscopy technique. *J. Biol. Chem.* **2021,** *297*.
(52) Liu, S.; Hoess, P.; Ries, J. Super-Resolution Microscopy for Structural Cell Biology. *Annual Review of Biophysics* **2022,** *51*, 301-326.
(53) Lelek, M.; Gyparaki, M.T.; Beliu, G.; Schueder, F.; Griffie, J.; Manley, S.; Jungmann, R.; Sauer, M.; Lakadamyali, M.; Zimmer, C. Single-molecule localization microscopy. *Nature Reviews Methods Primers* **2021,** *1*.
(54) Betzig, E.; Patterson, G.H.; Sougrat, R.; Lindwasser, O.W.; Olenych, S.; Bonifacino, J.S.; Davidson, M.W.; Lippincott-Schwartz, J.; Hess, H.F. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **2006,** *313*, 1642-1645.
(55) Rust, M.J.; Bates, M.; Zhuang, X.W. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **2006,** *3*, 793-795.
(56) Oi, C.; Mochrie, S.G.J.; Horrocks, M.H.; Regan, L. PAINT using proteins: A new brush for super-resolution artists. *Protein Sci.* **2020,** *29*, 2142-2149.
(57) Li, Y.; Mund, M.; Hoess, P.; Deschamps, J.; Matti, U.; Nijmeijer, B.; Sabinina, V.J.; Ellenberg, J.; Schoen, I.; Ries, J. Real-time 3D single-molecule localization using experimental point spread functions. *Nat. Methods* **2018,** *15*, 367-369.
(58) Sage, D.; Pham, T.A.; Babcock, H.*; et al.* Super-resolution fight club: assessment of 2D and 3D single-molecule localization microscopy software. *Nat. Methods* **2019,** *16*, 387-395.
(59) Schnitzbauer, J.; Strauss, M.T.; Schlichthaerle, T.; Schueder, F.; Jungmann, R. Super-resolution microscopy with DNA-PAINT. *Nat. Protoc.* **2017,** *12*, 1198-1228.
(60) Joseph, M.D.; Bort, E.T.; Grose, R.P.; McCormick, P.J.; Simoncelli, S. Quantitative Super-Resolution Imaging for the Analysis of GPCR Oligomerization. *Biomolecules* **2021,** *11*, 1503.
(61) Fischer, L.S.; Klingner, C.; Schlichthaerle, T.; Strauss, M.T.; Bottcher, R.; Fassler, R.; Jungmann, R.; Grashoff, C. Quantitative single-protein imaging reveals molecular complex formation of integrin, talin, and kindlin during cell adhesion. *Nat. Commun.* **2021,** *12*.
(62) Harwardt, M.L.I.E.; Schroder, M.S.; Li, Y.Q.*; et al.* Single-Molecule Super-Resolution Microscopy Reveals Heteromeric Complexes of MET and EGFR upon Ligand Activation. *Int. J. Mol. Sci.* **2020,** *21*.

(63) Sankaran, J.; Balasubramanian, H.; Tang, W.H.; Ng, X.W.; Rollin, A.; Wohland, T. Simultaneous spatiotemporal super-resolution and multi-parametric fluorescence microscopy. *Nat. Commun.* **2021,** *12*.
(64) Moller, J.; Isbilir, A.; Sungkaworn, T.*; et al.* Single-molecule analysis reveals agonist-specific dimer formation of micro-opioid receptors. *Nat. Chem. Biol.* **2020,** *16*, 946-954.
(65) Gwosch, K.C.; Pape, J.K.; Balzarotti, F.; Hoess, P.; Ellenberg, J.; Ries, J.; Hell, S.W. MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells. *Nat. Methods* **2020,** *17*, 217-224.


Single-Molecule Localization
Photobleaching Step Counting
dimer
tetramer
Intensity
Time
Förster Resonance Energy
Transfer Spectrometry
Frequency
1
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7
FRET Efficiency
Fluorescence Intensity
Fluctuation Spectrometry
Frequency
monomer
dimer
tetramer
Brightness

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