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Quantifying Membrane Protein Oligomerization with Fluorescence Cross-Correlation Spectroscopy

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Abstract

Fluorescence cross-correlation spectroscopy (FCCS) is an advanced fluorescence technique that can quantify protein-protein interactions in vivo. Due to the dynamic, heterogeneous nature of the membrane, special considerations must be made to interpret FCCS data accurately. In this study, we describe a method to quantify the oligomerization of membrane proteins tagged with two commonly used fluorescent probes, mCherry (mCH) and enhanced green (eGFP) fluorescent proteins. A mathematical model is described that relates the *relative cross-correlation value* (f_c) to the degree of oligomerization. This treatment accounts for mismatch in the confocal volumes, combinatoric effects of using two fluorescent probes, and the presence of non-fluorescent probes. Using this model, we calculate a ladder of f_c values which can be used to determine the oligomer state of membrane proteins from live-cell experimental data. Additionally, a probabilistic mathematical simulation is described to resolve the affinity of different dimeric and oligomeric protein controls.

Keywords

fluorescence correlation spectroscopy; fluorescence cross-correlation spectroscopy; dimerization; membrane protein dimerization; live cell fluorescence; FRET

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Author Information

M.J.K constructed the theory and algorithm. X.S. and Y.H. designed the FKBP system. Y.H. and R.L performed the sub-cloning of the FKBP controls. X.S. and S.K. performed the FCCS experiments. M.J.K and X.S. analyzed the data. M.J.K, X.S., and A.W.S. wrote the article. P.M. assisted M.J.K. in the development of the algorithm and took data.

Notes

Conflicts of interest: none

1. Introduction

Fluorescence correlation spectroscopy (FCS) is an established technique with many applications in biophysics. In the live-cell experiments, fluorescence fluctuations are measured as fluorescently tagged protein moieties diffuse in and out of a diffraction limited excitation volume. Correlation of these fluctuations ($G(\tau)$) gives information about concentration ($\langle N \rangle / V_{eff}$), mobility (τ_D), and oligomerization (η). A complimentary technique to FCS is fluorescence cross-correlation spectroscopy (FCCS), which correlates the diffusion of two spectrally separated species as they move through the excitation volume, denoted green for the shorter wavelength species and red for the longer wavelength species. In dual-color FCCS, excitation is typically achieved with two concentric beams, for instance from a 488 nm laser and a 561 nm laser. FCCS measures the concentration and mobility of the red-green co-diffusing species, which is indicative of the degree of association. The principle of FCCS has been described in several excellent reviews [1–8]. The focus of this paper is on the factors that affect the relative amplitude of the cross-correlation function. Several of these factors have been dealt with in several earlier reports that will be reviewed below. The goal of this paper is to combine each of the factors into a simple model that can be adapted to a variety of experimental conditions.

For applications of FCCS that use fluorescent proteins, their wide absorbance and emission spectra lead to spectral cross-talk which contaminates the cross-correlation data. In 2005, Müller et al. introduced pulsed interleaved excitation (PIE) as a modification to remove spectral crosstalk [9]. In PIE, picosecond laser pulses are temporally separated so that the fluorescence signals counted at each detector are gated corresponding to each excitation beam. In PIE-FCCS the cross-correlation function (CCF) is calculated from photons counted by detector A after 561 nm excitation with photons counted by detector B after 488 nm excitation. The red and green auto-correlation functions (ACFs) are calculated for fluorescence signal at detector A after 561 nm excitation and at detector B after 488 nm excitation respectively. Additional information can be obtained by measuring the donor fluorescence lifetime to determine the Förster resonance energy transfer (FRET) efficiency, which is accessible in PIE-FCCS with picosecond pulsed excitation and time-correlated single photon counting. We have used this technique to study dimerization of visual opsins, plexinA4 and other membrane receptors [10–12]. Recently, we used PIE-FCCS to study the organization of EGFR and found that cross-correlation can be used to resolve simple dimerization from the formation of larger multimers [13].

Special considerations must be made when interpreting the results of live cell measurements with fluorescent protein labels. Dual color excitation produces two wavelength-dependent volumes. Knowledge of the overlap and size of these volumes is essential for accurate determination of concentrations. Additionally, several factors lower (or dilute) the apparent cross-correlation which can lead to a misinterpretation of the data. The emergence of fluorescent protein (FP) fusions has contributed profoundly in the advancement of *in vivo* imaging, but incomplete maturation and dark states leadsto a significant population of receptors labeled with non-fluorescent FP. Non-fluorescent FP fusions can associate with other FP fusions, lowering the apparent concentration of co-diffusing species. When investigating homo-dimerization, same-color FP fusion dimers can bias the data because

they do not contribute to the CCF. Incomplete dimerization also lowers the apparent cross-correlation. Furthermore, protein oligomerization beyond simple dimerization leads to the formation of multiple diffusing species, which complicates the interpretation. Several approaches have been reported in the literature to deal with these experimental factors [14]. Rippe used DNA duplexes labeled with either fluorescein or rhodamine to investigate the NtrC-enhancer complex in solution [15]. It was noted that the duplex compositions follow a binomial distribution, which lowers the maximum binding ratio from 1 in the case of complete 1:1 binding of red and green probes to $\frac{1}{3}$ with binomial distribution. In a similar way, the binomial distribution was used to describe the integer accumulation of randomly-selected, singly-labeled molecules into a vesicle [4]. A thorough quantification of ligand binding with FCCS has also been detailed [16], including further corrections that account for the impact of the volume mismatch [17, 18]. While several groups have used FCCS to quantify heterodimeric interactions of cellular components [19–23], only a handful have used corrections for non-fluorescent labels and/or volume mismatch to quantify the apparent dissociation constant ($K_{D,app}$) [3, 14, 24–26]. Several recent studies have attempted to use FCCS data to quantify $K_{D,app}$ in the membrane, however, none of these papers have included corrections for the effective volume, the presence of non-fluorescent labels, and a binomial distribution due to homo-oligomerization [27–31]. In this publication, we collect all these corrections into a single model as it applies to a system with homo-oligomerization.

To compare the model with experimental data, we use a control system based on membrane-bound coiled-coil dimerization motif (GCN4). This control system has been reported in published work from our lab and includes a monomer control, a dimer control, and a multimer control [12, 13]. Here, we also constructed a new control system consisting of membrane anchored chimeras of the FK506 binding protein (FKBP), which dimerize upon stimulation of synthetic dimerizer. Using the model below we identify not only oligomer state of these control systems, but also their binding affinity in the live cell plasma membrane. These results demonstrate the ability of PIE-FCCS to identify homo-oligomerization and quantify the affinity of membrane protein-protein interactions in live cells.

2. Material & Methods

2.1 PIE-FCCS Instrument

Fluorescence measurements were collected on a customized inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan), which was described previously [10] (Fig. 1A). The 488 nm and 561 nm lasers beams were produced by a supercontinuum fiber laser with a repetition rate of 10 MHz (SuperK NKT Photonics, Birkerød, Denmark). Selection of the two beams from the white light source was achieved through a series of dichroic mirrors and clean-up filters (LL01-488-12.5, Semrock, Rochester, NY/LL02-561-12.5, Semrock, Rochester, NY). The 488 nm light was coupled into a 5 m single mode optical fiber while the 561 nm light was sent through a 15 m identical-core fiber, which introduced a time delay of about 50 ns. An additional dichroic (LM01-503-25, Semrock, Rochester, NY) was used to overlap the laser beams after exiting their respective fibers. A laser filter cube (zt488/561 rpc and zet488/561m, Chroma Technology, Bellows Falls, VT) reflected the overlapped excitation

light into the sample through a 100× oil-immersed objective (NA 1.49, Nikon, Tokyo, Japan). Fluorescence signal was collected as it passed back through the objective and was directed to one of the detection ports of the microscope. A 50 μm pinhole was positioned at the image plane, after which the light was collimated and then spectrally separated by a dichroic mirror (FF560-FDi01-25×36, Semrock, Rochester, NY). A final set of bandpass filters (FF01-520/44-25, Semrock, Rochester, NY / FF01-621/69-25, Semrock, Rochester, NY) were placed before two single photon avalanche diodes (SPAD, Micro Photon Devices, Bolzano, Italy). We assign the label **detector A** to the red signal (621±34 nm) and **detector B** to the green signal (520±22 nm). Single photons were time-tagged and time-resolved by a four-channel routed device (Picoharp 300, PicoQuant, Berlin, Germany) with a 32 ps timing resolution.

Time-correlated single-photon counting (TCSPC) data were time-gated such that photons at detector A within the 40 ns after the 561 nm pulse were assigned to $\mathbf{F_R(t)}$ and photons at detector B within 40 ns after the 488 nm pulse were assigned to $\mathbf{F_G(t)}$ (Fig. 1B). A multi-tau algorithm was used to calculate the auto and cross-correlation functions

$$G_{ij} = \frac{\langle \delta F_i(t) \cdot \delta F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle} \quad (1)$$

where i and j are either R or G so that for the two auto-correlations $i=j$ and for cross-correlation $i \neq j$ [32, 33]. Individual curves are averaged before fitting to an appropriate diffusion model using a non-linear least squares fitting algorithm.

2.2 Data Collection and Analysis

Transfected Cos-7 cells were grown on 35 mm uncoated #1 glass bottom dishes (P35G-1.0-20.C, MatTek Corporation, Ashland, MA) and changed to Opti-MEM I without phenol red (31985070, ThermoFisher Scientific, Waltham, MA) right before imaging. For the dimerizer assay, cells were incubated with AP20187 (HY-13992, MedChemExpress, Monmouth Junction, NJ) for 1 hour at a final concentration of 100 nM before imaging. The stage of the microscope is adapted with a stage-top incubator (Chamlide IC, Quorum Technologies, Guelph, Ontario) to keep cells at 37°C. The laser spot was focused on single cells in membrane areas so that we simultaneously measured the top and bottom membranes of the cell. For cell measurements, the 488 nm laser was set to a power of 300 nW while 561 nm was 800 nW as was measured before entering the back of the microscope. Data was collected at 5 × 10 s intervals for each cell and then averaged together for fitting. Single cell data were binned at 10 μs. The correlation data was fit to a single component 2D Brownian diffusion model with triplet blinking

$$G_i(\tau) = G(0) \cdot G_{Trip}(\tau) \cdot G_{Diff}(\tau)$$

$$G_i(\tau) = \frac{\gamma}{\langle N_i \rangle} \cdot \frac{1 - F + Fe^{-\tau/\tau_T}}{1 - F} \cdot \frac{1}{(1 + \tau/\tau_D)} \quad (2)$$

where i is either the R or G . The τ_T term is nominally the triplet relaxation time, but this time scale (~ 0.5 ms) is much longer than typical triplet state lifetimes, so we consider this time scale to reflect flickering into dark states, where F is the fraction of molecules in the dark state (typically 10–30%). N_i is the average number of diffusing species, γ is the correction factor used to quantify the Gaussian shape of the confocal volume and τ_D is the average dwell time of the diffusing species in the confocal volume. We assume γ to be 1 because measurements are taken at the substrate-sample interface; however, the value does not affect the quantification of f_c because it is a ratio and γ cancels out. The cross-correlation function is fit to the same model where F is assumed to be 0.

DNA standards were selected to calibrate the confocal volumes [1, 8, 9, 15, 34, 35]. A 40 nucleotide sequence was chosen with less than 50% G-C content [ACA AGC TGG AGT ACA ACT ACA ACA GCC ACA ACG TCT ATA T] with carboxy-tetramethylrhodamine (TAMRA) at the 5' end and 6-carboxyfluorescein (FAM) at the 3' end (Integrated DNA Technologies). An unlabeled complementary strand was also synthesized and the pair was annealed following the supplier's protocols with an excess of the unlabeled strand. The double-labeled, double-stranded DNA (TAMRA-40-FAM) was diluted using 10 mM TRIS buffer to a final concentration of 100 nM. Laser powers were set to 8 μ W and 5 μ W for the 488 nm and 561 nm lasers, respectively. Data collected was binned at 1 μ s bin times to compensate for the fast diffusion and fit to a 3D diffusion model with flickering

$$G_i(\tau) = \frac{\gamma}{\langle N_i \rangle} \cdot \frac{1 - F + Fe^{-\tau/\tau_T}}{1 - F} \cdot \frac{1}{(1 + \tau/\tau_D)} \cdot \frac{1}{\sqrt{1 + \tau/(\tau_D \kappa^2)}} \quad (3)$$

where κ is the eccentricity of the volume. We set κ to a constant value based on the refractive index of water and the numerical aperture of the objective [36, 37]. The uncorrelated background intensity was more four orders of magnitude lower than the signal intensity and thus has a negligible effect on the fit function.

2.3 Cell culture & transient transfection

Cell culture of Cos-7 cells was carried out with standard protocols using Dulbecco's Modified Eagle Medium (DMEM 1 $\times$ + GlutaMAX, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PenStrep, 15-140-122, Gibco, Waltham, MA). Transfection was done 1 day prior to imaging with Lipofectamine 2000 (11668019, ThermoFisher Scientific, Waltham, MA) according to the supplier's instructions. Src₁₆-eGFP/mCH, Src₁₃-GCN4-eGFP/mCH and Src₁₃-GCN4-EGFRk-eGFP/mCH plasmids were generously donated by Jay T. Groves (UC

Berkeley). The genes of FKBP1A were gifts from Carolyn Bertozzi (Addgene plasmid #20211, #20212 and #20213) and were sub-cloned into pAcGFP-N1 and pmCherry-N1 vectors with the Src membrane localization sequence inserted on the 5' end [38].

3. Fraction Correlated and Volume Correction

As fluorescently labeled proteins pass through the confocal volume, signal fluctuations are captured by single photon counting detectors. Each diffusing species contributes to the overall temporal correlation function. The amplitude of the correlation function is an average of all these diffusing species in the confocal volume over the acquisition period:

$$G_R(0) = \frac{\sum_{i=1}^n \epsilon_{R,i}^2 \langle N_i \rangle}{\left(\sum_{i=1}^n \epsilon_{R,i} \langle N_i \rangle \right)^2}$$

$$G_G(0) = \frac{\sum_{i=1}^n \epsilon_{G,i}^2 \langle N_i \rangle}{\left(\sum_{i=1}^n \epsilon_{G,i} \langle N_i \rangle \right)^2} \quad (4)$$

where $\epsilon_{R,i}$ is the average molecular brightness of species i in the red channel, $\epsilon_{G,i}$ is the average molecular brightness of species i in the green channel, and $\langle N_i \rangle$ is the average population of species i in the effective volume during the acquisition period [1, 2, 16, 22, 36]. The CCF amplitude is the summation of all the species that fluoresce in both channels divided by the product of total fluorescence signal found in each channel.

$$G_X(0) = \frac{\sum_{i=1}^n \epsilon_{R,i} \epsilon_{G,i} \langle N_i \rangle}{\left(\sum_{i=1}^n \epsilon_{R,i} \langle N_i \rangle \right) \left(\sum_{i=1}^n \epsilon_{G,i} \langle N_i \rangle \right)} \quad (5)$$

Using these correlation amplitudes and assuming a 1:1 stoichiometry, binding has been characterized by dividing the amplitude of the CCF by either of the ACFs such that $C_{RG}/C_{G,t} = G_X(0)/G_R(0)$ and $C_{RG}/C_{R,t} = G_X(0)/G_G(0)$ where R and G are the ACF amplitudes, X is the cross-correlation amplitude, C_{RG} is the apparent concentration of the co-diffusing species, and $C_{R,t}$ & $C_{G,t}$ are the apparent total concentrations of the red and green species, respectively [8, 14, 15]. We use a single metric, f_c or fraction correlated, as a relative measure of total oligomerization so that

$$f_c = \max \left(\frac{G_X(0)}{G_R(0)}, \frac{G_X(0)}{G_G(0)} \right) \quad (6)$$

where the fraction correlated would be 0 in a sample with no binding and 1 in a sample with complete 1:1 binding of the red and green species (Fig. 1D). The apparent populations that contribute to the three correlation amplitudes are volume dependent. In a system with perfect confocal volume overlap, the ratios of $G_X(0)/G_R(0)$ and $G_X(0)/G_G(0)$ should both be equal to 1 in the case of complete co-diffusion. However, the size of the confocal volume is diffraction limited by the wavelength of excitation

$$V_{conf} = \pi^{3/2} \omega^3 \kappa \quad (7)$$

where ω is the wavelength dependent lateral resolution and κ is the axial resolution divided by the lateral resolution. In dual color FCCS, two laser excitation results in two different volumes. In order to get a measure of the confocal volumes, a dually labeled 40 base pair DNA (TAMRA-40-FAM) is used as a 3D standard (Fig. 2A). The ratio of $G_X(0)/G_R(0)$ was found to have an average value of 0.85 while $G_X(0)/G_G(0)$ was 0.47, which are consistent with previous publications of similar samples [8, 9]. The difference between these ratios is due to the two different diffraction-limited beam waists. However, the non-ideal value of 0.85 is due to chromatic aberrations and imperfect overlap of the volumes. The apparent cross-correlation volume will be larger than the algebraic average of the two values due to the geometric displacements of the two confocal foci (Fig. 2B).[17, 31]

$$V_{X,app} = (\pi/2)^{3/2} (\omega_R^2 + \omega_G^2) \sqrt{(z_G^2 + z_R^2)} \cdot \exp\left(-\frac{(d_x^2 + d_y^2)}{\omega_X^2} - \frac{d_z^2}{z_X^2}\right) = \pi^{3/2} \omega_{X,app}^3 \kappa \quad (8)$$

The individual displacements in each axis are not known, therefore all the corrections are combined into one value, $\omega_{X,app}$. Assuming that the oligonucleotides are efficiently labeled, the relative cross-correlation amplitudes are related to the confocal volumes

$$\left(\frac{G_X(0)}{G_R(0)}\right)_{TAMRA-40-FAM} = \frac{[TAMRA-40-FAM]V_R}{[TAMRA-40-FAM]V_{X,app}} = \frac{V_R}{V_{X,app}} \quad (9)$$

$$\left(\frac{G_X(0)}{G_G(0)}\right)_{TAMRA-40-FAM} = \frac{[TAMRA-40-FAM]V_G}{[TAMRA-40-FAM]V_{X,app}} = \frac{V_G}{V_{X,app}}$$

where V_R & V_G are the confocal volumes from the 561 nm and 488 nm excitation respectively and V_X is the apparent volume of the cross-correlation due to imperfect overlap of the two volumes. The purity of the oligonucleotide was found to be about ~97% using capillary electrophoresis by the supplier with a small presence of minor peaks after the major oligonucleotide peak indicating residual impurities or contaminants like dust. Additionally, UV-VIS was used to quantify the ratio of each fluorescent probe to the total amount of oligonucleotide. On average, each strand was labeled with 1.4 and 1.2 photoactive

FAM and TAMRA tags, respectively, indicating that our assumption about labeling is valid for TAMRA-40-FAM. In this model, we calculate the average population of each possible diffusing species to be used in Eqs. (4) and (5). These theoretical values of the correlation amplitudes are volume corrected so that

$$f_c(Th) = \max \left[\left(\frac{G_X(0)}{G_G(0)} \right)^{Th} \left(\frac{V_G}{V_{X,app}} \right), \left(\frac{G_X(0)}{G_R(0)} \right)^{Th} \left(\frac{V_R}{V_{X,app}} \right) \right] \quad (10)$$

$$f_c(Th) = \max \left[\left(\frac{G_X(0)}{G_G(0)} \right)^{Th} (0.47), \left(\frac{G_X(0)}{G_R(0)} \right)^{Th} (0.85) \right]$$

where Th is the theoretical calculated value, and *TAMRA-40-FAM* is the standard sample used for obtaining the confocal volume overlap of the instrumental setup (Fig. 2C). This correction is applied to the calculations below. Strictly speaking, this correction applies to 3D samples. In a 2D sample, the displacement in the z axis is not a significant factor. Thus, $V_{x,app}$ is overestimated in this correction but is the best approximation without an adequate 2D positive control.

4. Theoretical Framework

4.1 Combinatorial dilution of f_c with two fluorescent tags

To measure protein homodimerization with PIE-FCCS, we label each half of the population with spectrally distinct fluorescent probes. A common fluorescent protein pair for these experiments is eGFP and mCH. Fluorescent proteins have the advantage of being genetically encodable, which means that single proteins are expressed as separate constructs and are thus allowed to natively encounter each other after translation. This process, however, also allows for the formation of same-label dimers, which lower the relative cross-correlation (Fig 1C) [12, 15]. Assuming a binomial distribution, the possible combinations of diffusing species (Fig 1C) dilutes the maximum cross-correlation value to a maximum of $\frac{1}{3}$ for obligate dimers [12, 15, 17]. Our aim here is to review how combinatorial dilution affects the maximum cross correlation for different oligomer sizes and setup the terminology used in the following sections for the fully corrected model.

In the experimental systems described above, membrane protein oligomers stochastically assemble from individual proteins, each labeled with one of two probes (i.e. eGFP and mCH). The number of labels is assigned the parameter $\mathbf{b}$, so for the eGFP/mCH system $\mathbf{b}=2$. For proteins that assembles into oligomers of size $\mathbf{a}$, the distribution of protomers within the oligomers follows binomial statistics. For monomers ($\mathbf{a}=1$) there are only two label possibilities, eGFP or mCH. For dimers ($\mathbf{a}=2$), there are four possible combinations, eGFP-eGFP, eGFP-mCH, mCH-eGFP, and mCH-mCH (Fig. 3A). However, two of these combinations are statistically identical, so we use a combinatorial factor to calculate the number of unique protomer combinations within an oligomer of size $\mathbf{a}$:

$$C_{a,b} = \binom{a+b-1}{a} = \binom{n}{m} = \frac{n!}{n!(n-m)!} \quad (11)$$

For a two-label, dimeric system, $a=2$ and $b=2$, so $n=3$, $m=2$, and $C_{a,b} = 3$. The population of each of these protomer combinations can be defined in terms of probabilities

$$\langle N_i \rangle = p_{mCH}^j p_{eGFP}^k w_i N_T \quad (12)$$

where w_i is the degeneracy of diffusing species, i , p_{mCH} and p_{eGFP} are the probabilities of finding a mCH and eGFP tagged receptor respectively, j and k are the integer number of mCH and eGFP in diffusing species i ($j+k = a$), and N_T is the total number of receptors. Initially, we assume that each receptor has a fluorescent tag so that

$$p_{mCH} + p_{eGFP} = 1 \quad (13)$$

Permutation is used to obtain the degeneracy of each diffusing species

$$w_i = \frac{a!}{j!k!} \quad (14)$$

Here, a is the number of oligomer size. The two homo-color dimers (eGFP-eGFP/mCH-mCH) have a degeneracy of one while the hetero-color dimer (eGFP-mCH) has a degeneracy of 2. Assuming equal expression of mCH- and eGFP-labeled proteins, the normalized populations are 0.25 and 0.5 for the two homo-color and hetero-color dimers respectively. The correlation amplitudes are also weighted by the molecular brightness of each species. Here we assume that the molecular brightness is an integer value of the monomer brightness so that

$$\varepsilon_{R,i} = j * \varepsilon$$

$$\varepsilon_{G,i} = k * \varepsilon \quad (15)$$

where ε is the molecular brightness of the monomeric species [14, 16, 39]. Measurements of molecular brightness have shown that dimers can vary from simple integer multiples of the monomer value. One possible cause for this change is the contribution of non-fluorescent FP, which is included later in our model, however we acknowledge that Eq. (14) is still an approximation of the average brightness [40]. Due to the advantages of PIE, we assume that the molecular brightness of each fluorescent probe in its non-dominant channel is zero, for instance, the molecular brightness of the eGFP-eGFP dimer in the red channel is zero.

Lastly, the total average population is canceled out when calculating f_c because it is a fractional value. In Table 1, cross-correlation values for dimer up to decamer are shown assuming equal expression of fluorescent moieties with and without volume correction. Cross-correlation values are proportional to the oligomerization state, which suggests that PIE-FCCS can be used to quantify the oligomer state of protein assemblies, especially at low oligomer sizes.

4.2 Inclusion of non-fluorescent protein fusions

Extensive study of fluorescent proteins has shown that both eGFP and mCH have incomplete maturation and can transition into dark states [14, 25, 41, 42]. The existence of non-fluorescent proteins increases the number of probes but does not affect the molecular brightness of the diffusing species so that the number of probes, b , increases to 3 and the definition of molecular brightness in Eq. (15) still applies. We include new parameters for the fraction of non-fluorescent of either FP

$$p_{mCH} = (1 - dR)p_{mCH} + (dR)p_{mCH}$$

$$p_{eGFP} = (1 - dR)p_{eGFP} + (dR)p_{eGFP} \quad (16)$$

$$(dR)p_{mCH} + (dR)p_{eGFP} = d$$

where dR and dG are the fraction of mCH and eGFP fluorescent proteins that are non-fluorescent respectively and the total fraction of non-fluorescent FP is represented by d . We can use these expressions to rewrite Eq. (12) to include all the new species with non-fluorescent receptors

$$\langle N_i \rangle = (p_{mCH}(1 - dR))^J (p_{eGFP}(1 - dG))^K d^n w_i N_T \quad (17)$$

where n is the integer number of non-fluorescent FP in diffusing species i . Degeneracy of each diffusing species will be modified by the presence of non-fluorescent FP so that

$$w_i = \frac{a!}{j!k!n!} \quad (18)$$

where the sum of j , k , and n is equal to a . Expanding the dimer example to include non-fluorescent FP, the number of combinations goes from three to six (Fig. 3B), where one of the species is the fully non-fluorescent dimer (not shown in Fig. 3B). Suppose mCH is estimated to be only 50% fluorescent, while eGFP is about 75% fluorescent [14, 41, 43–45]. In this case, f_c goes from 0.33 to 0.17, which brings the predicted value in close agreement to the experimental value seen for Src₁₃-GCN4 (see Fig. 4). We included calculated f_c values

for the different oligomer sizes based on the value of the non-fluorescent fraction. (**Error! Reference source not found.**). Note that these values are still proportional to oligomer size and give realistic boundaries for identifying the size of the oligomer based on f_c .

4.3 Incomplete Dimerization

Experimental measures of protein association have been used to either accept or deny the presence of dimerization or oligomerization. In reality, the cell membrane is a complex 2D fluid, where the affinity between membrane proteins can fall on a continuum of possible values, quantified by equilibrium constants. As a result, the relative populations of monomer or dimer are dependent on the total concentration of protein expressed in the cell. Therefore, it is important to incorporate the binding affinity into the model describing the cross-correlation values. We expand our model to include a simple two-state equilibrium so that

$$p_{state\ 1} + p_{state\ 2} = 1 \quad (19)$$

where $p_{state\ 1}$ and $p_{state\ 2}$ are the probabilities of finding a receptor in state 1 and state 2 respectively. We rewrite Eq. (17) to include the state of species i such that

$$\langle N_i \rangle = \frac{p_{state}}{a_{state}} (p_{mCH}(1 - dR))^j (p_{eGFP}(1 - dG))^k d^n w_i N_T \quad (20)$$

where p_{state} is the probability of finding species i in a_{state} such that a_{state} is an integer number of monomers. For the general case, the total number of diffusing species is the sum of the combinations available in the two states,

$$C_{a_{S1}, b} + C_{a_{S2}, b} = \binom{a_{S1} + b - 1}{a_{S1}} + \binom{a_{S2} + b - 1}{a_{S2}} \quad (21)$$

where a_{S1} and a_{S2} are the integer number of monomers in states 1 and 2 respectively and b is still the integer number of probes. Suppose a protein has incomplete dimerization such that only half of the total population dimerizes. In this case, the probabilities of finding a receptor in either a monomer or a dimer are equal ($p_{state\ 1} = p_{state\ 2} = 0.5$). Three new diffusing species are added to the six dimer species corresponding to the monomer of each probe (eGFP, mCH, non-fluorescent). Returning once again to the dimer example, the f_c value drops from 0.170 for a complete dimer to 0.094 for an incomplete dimer with moderate affinity (Fig. 3C). Therefore, a protein with high binding affinity or a small dissociation constant would have an f_c closer to that for a complete dimer ($f_c \approx 0.17$), while proteins with low affinity will have much lower f_c value ($f_c \approx 0.10$). Therefore within a reasonable range of affinities, f_c can be used to determine the strength of binding affinity for dimerization if no higher order oligomers are present.

5. Experimental Comparisons

5.1 Experimental PIE-FCCS

Correlation values were previously obtained for a series of control proteins (reproduced here with permission) (Fig. 4) [10, 12, 32]. A lipid-anchored peptide fused directly to a fluorescent protein is used as a negative control for cross-correlation (Src₁₆). This peptide is the single myristoylation anchor of the c-Src protein which has been shown to be monomeric in low densities on the cell surface [33, 46, 47]. The positive control for dimerization is the lipid-anchored peptide fused to the coiled-coil segment from GCN4 (Src₁₃-GCN4) [48]. Previous work has shown that the GCN4 leucine zipper has a high affinity for dimerization in solution [49]. PIE-FCCS studies confirmed that the GCN4 motif has high affinity for dimerization at a 2D surface where this motif is incorporated into a membrane bound protein [49, 50]. Lastly, a multimer control is constructed with the addition of the EGFR kinase domain fused between the GCN4 zipper and fluorescent protein (Src₁₃-GCN4-EGFRk). Multimerization is driven by the combination of the GCN4 dimerization motif and dimerization of kinase domain [13, 51]. Using the GCN4 control system, we constructed a ladder of f_c values to determine whether a membrane receptor was monomeric ($f_c=0.03$), dimeric ($f_c=0.14$), or multimeric with a size consistent with dimers, trimers, and tetramers ($f_c=0.24$) [12, 13].

In search of more rigorous oligomer controls, we created a new series of membrane proteins based on the FKBP motif for which the oligomerization can be chemically induced. FKBP is a 12 kD protein domain that can be induced to dimerize by treatment of a synthetic dimerizer (AP20187) [52]. We created three different FKBP constructs with different repeats of FKBP (1×FKBP, 2×FKBP and 3×FKBP). They were all fused to the lipid-anchored Src sequence so that the expressed proteins were localized to the plasma membrane. Either eGFP or mCH were fused to the C-terminus of the constructs as done previously for the GCN4 control constructs. Upon stimulation with the dimerizer (AP20187), induced dimerization of FKBP domains will occur. The repeat of FKBP domains enhances dimerization or leads to multimerization of the membrane-bound proteins. The 1×FKBP, 2×FKBP and 3×FKBP were transiently transfected, respectively, into Cos-7 cells which are subjected to PIE-FCCS measurements. The f_c values obtained from the experiments were summarized in Figure 5 where the medians of the f_c values were used to represent the oligomerization states. The results show that all three of the FKBP constructs have f_c values that are close to zero (Fig. 5, first to third columns). This means that the FKBP constructs are monomeric in live cell membranes. Upon treatment with the dimerizer (AP20187, hereby referred as AP), the f_c value of 1×FKBP increased to 0.11 (Fig. 5, fourth column), which means that 1×FKBP(+AP) has undergone dimerization in the cell membranes. However, this f_c value is lower than that observed with the other dimer control (Src₁₃-GCN4) which was around 0.14, suggesting incomplete dimerization. In the case of 2×FKBP, the repeat of the inducible-dimerization domain would enhance the affinity of dimerization or allow for oligomerization upon treatment of AP. As the fifth column of Figure 5 showed, the f_c value of 2×FKBP(+AP) is 0.16 which agrees with the value for a high affinity dimer. It is also possible that multimers can form when there is more than one dimerization domain in the protein. Treated 2×FKBP may exhibit a more complicated three state equilibrium which

is explored in the next section. Adding a third FKBP domain, 3×FKBP, did result in the formation of multimers. As shown in the sixth column of Figure 5, the f_c value of 3×FKBP(+AP) is 0.28 which agrees with the theoretical value for a complete trimer control (see Table 1, second column). Below, we investigate the possible oligomerization states of 3×FKBP(+AP).

5.2 Simulation of PIE-FCCS data

In this section, we explore the capability of the mathematical model to predict organization and binding affinity of the controls shown above. We will use a probabilistic approach to simulate cell data and then calculate the cross-correlation values. Comparison of the distribution of theoretical values with the experimental values will reveal information about the organizational state of each of the constructs, showing that the model can potentially elucidate not only oligomer state, but also the degree of association or binding affinity.

We define the binding affinity by the dissociation constant. *In vivo* dimerization is the result of monomer-dimer equilibrium.

$$K_d = \frac{[Monomer]^2}{[Dimer]} \quad (22)$$

In order to obtain information about the dissociation constant of the dimer control constructs, we redefine Eq. (22) in terms of parameters from the model

$$K_d = \frac{[Mon]^2}{[Dimer]} = \frac{(p_{state\ 1} C_T)^2}{\left(\frac{p_{state\ 2} C_T}{2}\right)} = \frac{2C_T(1 - p_{state\ 2})^2}{p_{state\ 2}} \quad (23)$$

where $p_{state\ 1}$ and $p_{state\ 2}$ are the probabilities of finding a receptor in a monomer and a dimer respectively, and C_T is the total concentration of receptors. Correlation amplitudes in Eq. (4) are re-written in terms of concentrations

$$G_R(0) = \frac{\sum_{i=1}^n \epsilon_{R,i}^2 C_i}{\left(\sum_{i=1}^n \epsilon_{R,i} C_i\right)^2 N_A V_R}$$

$$G_R(0) = \frac{\sum_{i=1}^n \epsilon_{G,i}^2 C_i}{\left(\sum_{i=1}^n \epsilon_{G,i} C_i\right)^2 N_A V_G} \quad (24)$$

Where C_i is the concentration of species i , N_A is Avogadro's number, and V_R & V_G are the confocal volumes from the 561 nm and 488 nm excitation respectively. The cross-correlation amplitude can be modified similarly

$$G_X(0) = \frac{\sum_{i=1}^n \epsilon_{R,i} \epsilon_{G,i} C_i}{\left(\sum_{i=1}^n \epsilon_{R,i} C_i\right) \left(\sum_{i=1}^n \epsilon_{G,i} C_i\right) N_A V_X} \quad (25)$$

where V_X is the apparent volume of the cross-correlation due to imperfect overlap of the two volumes. Using the structure of Eq. (20), the concentration of species i is defined

$$C_i = \frac{p_{state}}{a_{state}} (p_{mCH}(1 - dR))^j (p_{eGFP}(1 - dG))^k d^n w_i C_T \quad (26)$$

where C_T is the average total concentration of receptors in the confocal volume. In this approach, we simulate experimental values by using a matrix of parameters that are randomly generated within acceptable ranges. The average total concentration used for 100 theoretical cells was 100 ± 20 nM. In order to quantify incomplete maturation of our FPs, we performed UV-VIS measurements of histidine tagged eGFP-mCH tandem constructs purified in *E. coli*. The molar absorptivity of the protein (ϵ_{280}) was calculated to be $\sim 56,000$ $M^{-1}cm^{-1}$ based on the amino acid sequence [53], while the molar absorptivities of mCH and eGFP were assumed to $72,000$ $M^{-1}cm^{-1}$ and $55,000$ $M^{-1}cm^{-1}$, respectively, based on literature [54, 55]. Samples were diluted using $1 \times$ PBS and analyzed using an Agilent Cary 100 UV-Vis spectrophotometer. Using a range of samples ($5 \mu M - 15 \mu M$), the average fraction of non-fluorescent mCH was $\sim 52\%$, while the fraction of non-fluorescent eGFP was $\sim 40\%$. In our mammalian system, we assume mCH is also about 50% fluorescent as was observed in other systems [14, 25], in contrast we assume the maturation of eGFP is higher than what we observed from bacterial expression to be about 75% because maturation of eGFP has been reported to range from 50% to 100% in mammalian cell lines [14, 41, 43–45]. Therefore, the maturation values of eGFP and mCherry were randomized to have an average values of $75 \pm 13\%$ and $50 \pm 20\%$, respectively, and the apparent ratio of red to green ($G_G(0)/G_R(0)$) was within the range of 2:3 to 3:2. The fraction of state 2, $p_{state\ 2}$, was calculated based on the relationship defined in Eq. (23). The theoretical f_c is calculated by dividing Eq. (25) by the minimum of the two ACF amplitudes in Eq. (24). Ratios of the confocal volumes obtained from TAMRA-40-FAM are used for the apparent confocal volumes. Cross correlation values are calculated for dissociation constants from 5 nM up to 5 μM (not shown). Dimerization drops below 10% for dissociation constants larger than 1 μM . At a dissociation constant of 500 nM, the average f_c value is 0.05 which corresponds to about 20% dimerization.

Comparison with our simulated results indicate that Src₁₃-GCN4 has a dissociation constant of ~ 20 nM (Fig. 6a). This is lower than the dissociation constant of the GCN4 leucine zipper in solution, which is ~ 8 nM according to Nikolaev et al. [49]. However, additional work indicates that a concentration of at least 10 μM was needed to create stable dimers [50]. The lower apparent dissociation constant may also be due to steric hindrance of the dimerization interface when anchoring the motif to the membrane [56]. Therefore, we suggest that the availability of proteins and interaction interfaces in the membrane leads to a lowering of

dissociation constant. 1×FKBP(+AP) had an f_c value of 0.11, which matches the calculated f_c value for a dimer with a dissociation constant of 75 nM (Fig. 6b), which is in agreement with the nanomolar affinity seen for FKBP in other work [57]. At this affinity only about 50% of the receptors are part of a dimeric complex.

The addition of a second FKBP domain increases the cross correlation to 0.16. As indicated above, this may be due to a shift in the dimer binding affinity or appearance of a multi-state equilibrium. Three-state equilibrium requires the determination of two dissociation constants. For monomer-dimer-trimer equilibrium, the first equilibrium is the same as Eq. (24) while the second K_d can be defined as

$$K_{D,2} = \frac{[Monomer][Dimer]}{[Trimer]} = \frac{(p_{state1}C_T)\left(\frac{p_{state2}C_T}{2}\right)}{\left(\frac{p_{state3}C_T}{3}\right)} \quad (27)$$

where p_{state3} is the fraction of receptors participating in a trimer and the sum of all the fractions is equal to one. Similarly, the second K_d for monomer-dimer-tetramer equilibrium is

$$K_{D,2} = \frac{[Dimer]^2}{[Tetramer]} = \frac{\left(\frac{p_{state2}C_T}{2}\right)^2}{\left(\frac{p_{state4}C_T}{4}\right)} \quad (28)$$

where p_{state4} is the fraction of receptors participating in a tetramer. In the three-state equilibria, monomer-dimer dissociation constant for 2×FKBP(+AP) is assumed to be equivalent to that obtained for the single FKBP construct (75 nM). In order to compare these possible configurations, Figure 6c shows a comparison of 2×FKBP(+AP) with calculated f_c values for monomer-dimer equilibrium, monomer-dimer-trimer equilibrium and monomer-dimer-tetramer equilibrium (Fig. 6c, columns 2 through 4). Visual comparison of these theoretical values indicates that the distribution of values becomes larger for more complicated equilibria, which is more consistent with the experimental data seen in the first column. The last column shows the distribution of calculated f_c values for monomer dimer equilibrium based on a K_d of 5 nM with modified parameters. In this simulation, the average concentration is 200 ± 100 nM and the apparent population ratio ($G_R(0)/G_G(0)$ or $G_G(0)/G_R(0)$) is within 1:4. Modification of the parameter matrix results in a spread of values that is visually similar to that observed for the experimental data. The fractional values of each oligomer (monomer, dimer, trimer, and tetramer) is shown for each equilibria in Table 4 (A: monomer-dimer, B: dimer-tetramer, C: monomer-dimer-trimer & D: monomer-dimer-tetramer). For 2×FKBP(+AP), ~40% of the receptors would be monomeric if in a three-state equilibrium. However, previous work has shown that the addition of the second FKBP simply results in a higher affinity for dimerization with no evidence for higher order oligomers [58]. This is consistent with the molecular brightness observed for 2×FKBP(+AP)

which is twice the amount of the monomeric form of the FKBP constructs (data not shown). Therefore, we conclude that induced 2×FKBP results in dimerization with a high affinity ($K_d \sim 5$ nM).

Continuing the multi-state equilibrium analysis, we analyze the two multimer controls, Src₁₃-GCN4-EGFRk and 3×FKBP(+AP). We use the knowledge obtained from the previous simulations to guide determination of the parameters in the three-state equilibria. For Src₁₃-GCN4-EGFRk, the dissociation constant for monomer-dimer equilibrium is assumed to be 20 nM based on the simulated data for the Src₁₃-GCN4 construct. Because ~30% of the population was still in a monomeric form, the addition of the kinase domain can cause the formation of trimer or tetramers. Based on our simulations, monomer-dimer-trimer equilibrium would result in a trimer percentage of ~60% ($K_d \sim 10$ nM), while monomer-dimer-tetramer equilibrium ($K_d \sim 50$ nM) would only have ~35% formation of tetramers (Fig. 6d). Due to the availability of monomers after GCN4 dimerization, oligomerization of Src₁₃-GCN4-EGFRk could also be a combination of all four states (monomer, dimer, trimer, and tetramer), which is beyond the scope of this work. For 3×FKBP(+AP) we make the assumption that it does not form trimers based on the high dimerization affinity of the previous tandem construct. Therefore, 3×FKBP(+AP) is compared to simulated data with monomer-dimer-tetramer and dimer-tetramer equilibria (Fig. 6e). Both equilibria result in a ~60% formation of tetramers. In fact, for the three-state equilibrium, the fraction of tetramer is equal to the fraction of dimer formed for the original 1×FKBP(+AP) construct. This evidence strongly suggests that the triple tandem FKBP construct forms tetramers with relatively high affinity. Additionally it is apparent that 3×FKBP exhibits a higher affinity for oligomerization than Src₁₃-GCN4-EGFRk. In Figure 6f, each construct is plotted relative to their degree of oligomerization and affinity. As a reference, the monomeric control, Src₁₆ is placed in the bottom left corner of the plot. The three dimer controls, 1×FKBP(+AP), Src₁₃-GCN4, and 2×FKBP(+AP), are shown in a row with increasing dimerization affinity. The fraction of dimer calculated by the dissociation constant is used to quantify the dimerization affinity (see Table 4, column A for each construct). Src₁₃-GCN4-EGFRk is placed vertically closer to 3×FKBP(+AP) than to the dimer controls to indicate the possible mix of monomer, dimer, trimer and tetramers. The horizontal placement is an average of the fractions of trimer and tetramer obtained for the two equilibria. Lastly, 3×FKBP(+AP) is placed directly above 1×FKBP(+AP) at a vertical level indicating the formation of tetramers.

6. Concluding Remarks

In this study, we have shown that the f_c value can be used as a metric for determining the oligomer state and affinity of membrane protein-protein interactions. Using statistics and our knowledge of the systems, we calculated the expected f_c values for different oligomer sizes and affinity. Accounting for the non-fluorescent fractions of mCH and eGFP (50% and 25%) and including a volume correction for our instrumental setup, a complete dimer control should have an f_c value of ~0.17, which is within range of experimental values observed for previous dimeric protein constructs (Plexin $f_c = 0.16$ and red cone opsin $f_c = 0.19$) [11, 12]. Our dimer controls, Src₁₃-GCN4, 1×FKBP(+AP), and 2×FKBP(+AP), show a distribution of f_c values, 0.14, 0.11 and 0.16 respectively. We conclude that treated 2×FKBP has the highest affinity for dimerization, with a little to none of the proteins in the monomeric form.

Additionally, Src₁₃-GCN4 and treated 1×FKBP can be used as a scale for dimerization affinity. Using our mathematical treatment of f_c , we conclude that on average ~70% of Src₁₃-GCN4 and ~50% of treated 1×FKBP dimerize. In order to relate these values to a dissociation constant, a mathematical simulation of cell data is performed assuming differing degrees of affinity. Our model indicates that these dimer controls have a range of dissociation constants from 5 nM for 2×FKBP(+AP) to 20nM for Src₁₃-GCN4 to 75 nM for treated 1×FKBP. Therefore, using our model, f_c values can quantify the dissociation constant of different receptors over a modest range of binding affinities. Additionally, we calculated the expected f_c value for dimers, trimers, tetramers, up to an oligomer cluster of 10. This scale can be used in future studies to identify the degree of oligomerization of other membrane proteins. The multimer controls presented here, Src₁₃-GCN4-EGFRk and 3×FKBP(+AP), have mean f_c values of 0.24 and 0.28. These values are significantly higher than values attainable for fully dimerized receptors. Using the information obtained for the dimer controls, simulated data for multiple equilibria is used to determine the oligomer state of each multimer. The model outlined in this work quantitatively resolves the relationship between cross-correlation and the oligomerization state of membrane proteins.

7. Other Considerations

The mathematical treatment of FCCS data presented here is adaptable to many other systems. We showed that a single mathematical model can be used to describe a system with homo-oligomerization taking into account several significant correction factors. There are too many unknown parameters to fit the model to an experimental data set. However, with reasonable estimates of the parameters, comparison with the f_c distribution from the experiment and the simulated data can revealed information about the size of oligomer and the binding affinity of dimers for cells expressing mCH and eGFP tagged fusion proteins. Simple modifications can be included to use this calculation for any set of fluorescent probes. In addition, our calculations are compared to data from a system which utilizes PIE-FCCS, but the generalized model can be used to calculate values for instruments without PIE using the appropriate crosstalk corrections. Lastly, we focused this analysis on homo-oligomerization of membrane proteins; nonetheless, with some modifications this approach can be used for determination of hetero-oligomerization and ligand binding.

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- We address the impact of oligomerization and binding affinity on cross-correlation.
- A new mathematical model is derived to describe the cross-correlation function amplitude.
- The model accounts for the influence of protein combinations and non-fluorescent probes.
- Simulated data is used to elucidate K_D and oligomer size of membrane proteins.

Fig. 1.

Schematic of PIE-FCCS measurements and analysis. **(A)** Simple representation of the optical path used to illuminate samples. In short, a pulsed white light is used to source the two excitation beams. Beams are sent through optical fibers of different lengths to introduce a 50 ns delay. The overlapped beams go through the objective to illuminate the sample in the confocal volume. Fluorescence passes back through the objective and is spectrally separated into two detectors ($F_R(t)$ & $F_G(t)$). **(B)** TCSPC at each detector is achieved when the probability of detecting a photon during the sync pulse is either 0 or 1. Photons are binned according to their arrival time in relation to the sync pulse. **(C)** Homo-dimerization of red, green and non-fluorescent FP proteins results in several possible diffusing species. Relative fluorescence contributions of each species in detectors A and B are shown. **(D)** After correlation, information about the diffusion (τ_D & D_{eff}), concentration ($\langle N \rangle / V_{eff}$), and oligomerization (f_c) are extracted by fitting the correlation function to a diffusion model.

Fig. 2.

Dual labeled oligonucleotides are used for calibration of our PIE-FCCS instrument. **(A)** Representative FCCS data for TAMRA-40-FAM shows high cross-correlation. **(B)** Due to chromatic aberrations, the foci of the two confocal volumes are not perfectly concentric. The relative cross-correlation values, $G_X(0)/G_R(0)$ & $G_X(0)/G_G(0)$, of TAMRA-40-FAM are equal to the ratio of the effective volumes. **(C)** These ratios are used for the volume overlap corrections of the theoretical values of f_c calculated with the model.

Fig. 3.

Schematic of combinatorial effects on the f_c value. (A) Red and green labeled homodimers mix with binomial distribution. (B) Due to FP maturation, some proteins are labeled with non-fluorescent FP which increases the number of diffusing species. For simplicity, all fully non-fluorescent combinations are not depicted. (C) In live cells, a dimeric receptor likely exhibits equilibrium between monomeric and dimeric species. The correlation amplitudes are written out including all the diffusing species affecting their calculation. Example correlation curves are shown assuming equal FP expression in the cell where $G_R(\tau)$ is red, $G_G(\tau)$ is green, and $G_X(\tau)$ is blue. The scales of $G(\tau)$ and τ were arbitrarily determined by assuming there were on average 40 proteins with an average dwell time of 50 ms. For simplicity, example curves were produced using a 2D Brownian diffusion model without triplet relaxation. The green FP is assumed to be 75% fluorescent, while the red is assumed to be only 50% fluorescent as seen in literature for eGFP and mCH. For the f_c of the incomplete dimer, the fraction of proteins participating in a monomer is equal to the fraction participating in a dimer ($p_{stateF} = p_{stateF} = 0.5$).

Fig. 4.

Cross correlation values for the GCN4 controls. The constructs are displayed in decreasing oligomerization. (Data was previously obtained and reproduced here with permission [3]). The average f_c values were 0.24, 0.14 and 0.02, respectively.

Fig. 5.

f_c values of the FKBP controls obtained from PIE-FCCS measurements in live Cos-7 cells.

The three columns on the left side show the results of FKBP constructs without their dimerizer to induce interaction. The three columns on the right side show the results of FKBP constructs treated with the dimerizer (AP20187) which induces dimerization/oligomerization of the proteins. Each data point was the average of five 10 s FCS measurements performed on one cell. The purple numbers beside the box plots are the median values of the f_c values. The grey numbers on top the plots are the total number of cells used. The one-way ANOVA tests were performed to obtain the p values (****: $p < 0.0001$, **: $p < 0.01$, ns: not significant).

Fig. 6.

Comparison of experimental f_c data versus simulated data for different equilibrium. (A) Using the analysis outlined in Section 5.2, 1xFKBP(+AP) cross-correlation values are comparable to expected values for a receptor with a monomer-dimer equilibrium of 75 nM. At this level of affinity ~50% of the population will be present in dimers. (B) With higher cross-correlation values, the theoretical dissociation constant of Src₁₃-GCN4 is 20 nM, which indicates a higher affinity for dimerization compared to 1xFKBP(+AP). (C) Because of the higher average f_c value of 2xFKBP (0.16), multiple possible equilibrium states are possible. The calculated cross-correlation values for monomer-dimer (1st column), monomer-dimer-trimer (2nd column), and monomer-dimer-tetramer (3rd column) equilibria are simulated for the standard conditions (stated below). For the three state equilibria, the dissociation constant for monomer-dimer is assumed to be equal to that found for the 1st FKBP domain (75nM). The 4th column shows a wider spread of values for monomer-dimer equilibrium with a dissociation constant of 5 nM where the conditions are modified (below) to show that the spread in the experimental f_c values can be due to a large amount of cell variability. (D) The f_c value of Src₁₃-GCN4-EGFRk is too high to have monomer-dimer equilibrium, therefore a three state monomer-dimer-trimer and monomer-dimer-tetramer were simulated. The monomer-dimer equilibrium is assumed to be equal to what was obtained for the GCN4 zipper domain (20 nM). (E) Since 2xFKBP(+AP) was found to have such a high dimerization affinity, experimental 3xFKBP(+AP) data was compared to monomer-dimer-tetramer and dimer-tetramer equilibria. The percentage of dimer and

tetramer in both possible states are very similar (see Table 4). (F) Each construct was plotted based on its most probable oligomer size and binding affinity. Relative binding affinity was based on the percentage of receptors participating in the respective oligomer. IE 1×FKBP(+AP), Src₁₃-GCN4, and 2×FKBP(+AP) are all inline with each other (dimers) with varying degree of affinity (53%, 72%, and 85% dimer respectively).

TABLE 1

Calculated f_c Values^{*}

Oligomer State	2	3	4	5	6	7	8	9	10
Fully fluorescent	f_c	0.333	0.500	0.600	0.666	0.714	0.750	0.777	0.818
	$f_{c, Vcor}$	0.283	0.425	0.510	0.567	0.607	0.637	0.661	0.680
inclusion of non-fluorescent populations [†]	f_c	0.273	0.429	0.529	0.600	0.652	0.692	0.724	0.750
	$f_{c, Vcor}$	0.170	0.283	0.364	0.425	0.472	0.510	0.541	0.567

^{*} Values are based on the model outlined in Section 4.1 & assume $p_T = p_g = 0.5$

f_c is the theoretical value

$f_c, Vcor$ is the theoretical value based on the volume correction for our instrument

[†] mCherry is assumed to be 50% non – fluorescent & 25% of eGFP is assumed to be non – fluorescent

Table 2

Generalized Calculations

	2 color oligomer	2 color oligomer + non-fluorescent	2 color oligomer + non-fluorescent + equilibrium
<i>Assumptions</i>	$p_{mCH} + p_{eGFP} = 1^a$	$p_{mCH}(1 - dR) + p_{eGFP}(1 - dG) + d = 1^d$ $d = p_{mCH} dR + p_{eGFP} dG$	$\rightarrow$ $\rightarrow$ $p_{s1} + p_{s2} = 1^f$
$\langle N_i \rangle_{norm}$	$p_{mCH}^j p_{eGFP}^k w_i^b$	$(p_{mCH}(1 - dR))^j (p_{eGFP}(1 - dG))^k d^n w_i^e$	$\frac{p_{state}}{a_{state}} (p_{mCH}(1 - dR))^j (p_{eGFP}(1 - dG))^k d^n w_i^g$
<i>Degeneracy</i> (w_i)	$w_i = \frac{a!}{j!k!}^c$	$w_i = \frac{a!}{j!k!n!}$	$\rightarrow$
<i>Molecular Brightness</i> (e)	$e_{R,i} \cong j * e$ $e_{G,i} \cong k * e$	$\rightarrow$ $\rightarrow$ (brightness unaffected by non-fluorescent FP)	$\rightarrow$ $\rightarrow$ (brightness unaffected by equilibrium)

^a p_{mCH} & p_{eGFP} are the fractions of mCherry and eGFP expressed in the cell respectively

^b j & k are the number of mCherry and eGFP tags in species i respectively

^c a is the number of receptors participating in species i

^d dR & dG are the fractions of non – fluorescent mCherry and eGFP respectively

^e n is the number of non – fluorescent tags in species i

^f p_{s1} & p_{s2} are the probabilities of finding a receptor in state 1 and state 2 respectively

^g p_{state} the probability of finding a receptor in that state and a_{state} is the integer number of monomers in that state

Table 3

Generalized Equilibrium Equations

	Equilibrium	Equilibrium General K_D	Assumption	K_D Modified
<i>Monomer - Dimer</i>	$2M \leftrightarrow M_2$	$\frac{[M]^2}{[D]}$	$p_M + p_D = 1$	$\frac{(p_M^{C_T})^2}{\left(\frac{p_D^{C_T}}{2}\right)}$
<i>Dimer - Tetramer</i>	$2M_2 \leftrightarrow M_4$	$\frac{[D]^2}{[Tet]}$	$p_D + p_{Tet} = 1$	$\frac{\left(\frac{p_D^{C_T}}{2}\right)^2}{\left(\frac{p_{Tet}^{C_T}}{4}\right)}$
<i>Monomer - Dimer - Trimer</i>	$2M \leftrightarrow M_2$	$\frac{[M]^2}{[D]}$	$p_M + p_D + p_{Tri} = 1$	$\frac{(p_M^{C_T})^2}{\left(\frac{p_D^{C_T}}{2}\right)}$
	$M + M_2 \leftrightarrow M_3$	$\frac{[M][D]}{[Tri]}$		$\frac{(p_M^{C_T})\left(\frac{p_D^{C_T}}{2}\right)}{\left(\frac{p_{Tri}^{C_T}}{3}\right)}$
<i>Monomer - Dimer - Tetramer</i>	$2M \leftrightarrow M_2$	$\frac{[M]^2}{[D]}$	$p_M + p_D + p_{Tet} = 1$	$\frac{(p_M^{C_T})^2}{\left(\frac{p_D^{C_T}}{2}\right)}$
	$2M_2 \leftrightarrow M_4$	$\frac{[D]^2}{[Tet]}$		$\frac{\left(\frac{p_D^{C_T}}{2}\right)^2}{\left(\frac{p_{Tet}^{C_T}}{4}\right)}$

Table 4

Distribution of receptors into various states

	1×FKBP(+)/AP				Src13-GCN4				2×FKBP(+)/AP				Src13-GCN4-EGFRk				3×FKBP(+)/AP				
	A	B	C	D	A	B	C	d	A	B	C	D	A	B	C	D	A	B	C	D	
(f _c)	0.11	-	-	-	0.14	-	-	-	0.16	-	-	0.16	0.16	-	-	0.23	0.23	-	0.27	-	0.28
<hr/>																					
Mon	47	-	-	-	28	-	-	-	15	-	-	40	42	-	-	16	22	-	-	-	10
Dim	53	-	-	-	72	-	-	-	85	-	-	35	43	-	-	25	43	-	35	-	37
Tri	-	-	-	-	-	-	-	-	-	-	-	25	-	-	-	59	-	-	-	-	-
Tet	-	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-	35	-	65	-	53
<hr/>																					
K _D	75	-	-	-	20	-	-	-	5	-	-	75	75	-	-	20	20	-	2	-	5
												70	125	-	-	10	50	-	-	-	25