Understanding the FRET Signatures of Interacting Membrane Proteins

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FRET is an indispensable experimental tool for studying membrane proteins. Currently, two models are available for researchers to determine the oligomerization state of membrane proteins in a static quenching FRET experiment: the model of Veatch and Stryer, derived in 1977, and the kinetic theory-based model for intraoligomeric FRET, derived in 2007. Because of confinement in two dimensions, a substantial amount of FRET is generated by energy transfer between fluorophores located in separate oligomers in the two-dimensional bilayer. This interoligomeric FRET (also known as stochastic, bystander, or proximity FRET) is not accounted for in either model. Here, we use the kinetic theory formalism to describe the dependence of the FRET efficiency measured in an experiment (i.e., the “total apparent FRET efficiency”) on the interoligomeric FRET due to random proximity within the bilayer and the intra-oligomeric FRET resulting from protein-protein interactions. We find that data analysis with both models without consideration of the proximity FRET leads to incorrect conclusions about the oligomeric state of the protein. We show that knowledge of the total surface densities of fluorophore-labeled membrane proteins is essential for correctly interpreting the measured total apparent FRET efficiency. We also find that bulk, two-color, static quenching FRET experiments are best suited for the study of monomeric, dimerizing, or dimeric proteins but have limitations in discerning the order of larger oligomers. The theory and methodology described in this work will allow researchers to extract meaningful parameters from static quenching FRET measurements in biological membranes.

Many recent investigations of the function and interaction mode of membrane proteins in the plasma membrane, such as G-protein-coupled receptors and receptor tyrosine kinases, have been enabled through the use of fluorescence microscopy (1–7). FRET, a quantum mechanical transfer of energy from an optically excited donor to an acceptor over distances usually limited to about 10 nm, is a property of certain pairs of fluorophores, like the fluorescent proteins mTurquoise and YFP (8). These fluorescent proteins are attached to membrane proteins of interest, and when these tagged proteins dimerize or oligomerize, intraoligomeric FRET can occur from the excited donor to a nearby acceptor, resulting in quenched donor emission and enhanced emission from the acceptor (9–11). Due to the short distances over which FRET can occur (∼1–10 nm), FRET is often referred to as a “molecular ruler” (12–17). However, FRET between donor- and acceptor-tagged membrane proteins does not exclusively occur intraoligomerically (i.e., within dimers or oligomers). Non-negligible interoligomeric proximity FRET (also known as stochastic or bystander FRET) can also occur between donor- and acceptor-labeled membrane proteins that belong to different oligomers (or monomers) as a result of fluorophore confinement in two dimensions and total surface density (4, 18, 19). This interoligomeric FRET occurs simply because membrane proteins belonging to different oligomers find themselves within close proximity (i.e., within ∼10 nm) in the bilayer. The magnitude of the proximity FRET is known to be a function of the membrane protein oligomerization state and the acceptor surface density but is not well understood from a theoretical standpoint.

The function of receptors in the cell membrane is often modulated by their lateral association in the membrane into dimers or higher order oligomers (20–22). Thus, the association state of a membrane protein (monomer, dimer, oligomer, etc.) is of primary interest. Bulk, static-quenching FRET is often the method of choice in the study of the association state (23–26). Parameters of interest in such studies are the number of proteins in a functional oligomer and the value of the intrinsic FRET or pairwise FRET efficiency (27–29). The latter depends on the distance between the fluorophores in the oligomer and provides insights into the architecture of the oligomer.

Two models are currently in use for the analysis of static quenching FRET experimental data (28, 30). The first is the Veatch and Stryer model (31), derived in 1977, which is often utilized in semiquantitative experiments to determine the oligomeric state of the membrane proteins. In these experiments, the donor/acceptor ratio is known, but the total surface density of donor- and acceptor-labeled receptors is unknown or is not utilized in the analysis. The second model is the theory of intraoligomeric FRET based on the kinetic theory formalism, proposed in 2007 (32), which derives its relations from an explicit
consideration of rates of energy transfer and donor/acceptor combinatorics in ensembles of labeled oligomers. The full kinetic theory of intraoligomeric FRET can be greatly simplified by assuming a single donor-acceptor distance in the oligomer, and this simplified theory is often used to analyze fully quantitative FRET experiments where the total surface densities of donor- and acceptor-labeled membrane proteins are known or measured but the oligomeric state is unknown and is of interest (32). Neither the model of Veatch and Stryer nor the kinetic theory of intraoligomeric FRET account for the interoligomeric proximity FRET that occurs with labeled membrane proteins.

Here we use the kinetic theory formalism to describe the theoretical dependence of the total FRET efficiency measured in a static quenching FRET experiment, the “total apparent FRET efficiency,” on the interoligomeric proximity FRET and on the intraoligomeric FRET due to protein-protein interactions. We then utilize computer simulations of the measured total apparent FRET efficiency to study the consequences of ignoring proximity FRET when interpreting experimental results with both the model of Veatch and Stryer and the kinetic theory of FRET-based model. We also study the limitations of these models in deducing the oligomerization state of labeled membrane proteins. We find that data analysis without consideration of proximity FRET leads to incorrect conclusions about the oligomeric state of the protein with both models. We show that knowledge of the total surface densities of fluorophore-labeled membrane proteins is essential for the correct interpretation of the measured total apparent FRET efficiency because only the kinetic theory formalism is able to extract the proper oligomerization state. We find that bulk, two-color static quenching FRET experiments are best suited for the study of monomeric, dimerizing, or dimeric proteins and that there are limitations in the determination of higher order oligomerization states. Further, we find that for low values of intrinsic FRET in the oligomer, even dimerization may be difficult to discern from higher order oligomerization in a bulk static quenching FRET experiment, despite the correct account of proximity FRET.

Results

Theory of Total Apparent FRET Efficiency with Non-negligible Proximity FRET—The total (donor-quenched) apparent FRET efficiency, $E_{\text{app}}$, is defined as the ratio of the rate of resonance energy transfer, $k_{\text{RET}}$, to the overall rate of all donor de-excitation pathways, including FRET, according to Equation 1.

$$E_{\text{app}} = \frac{k_{\text{RET}}}{k_D + k_{\text{RET}}} \quad (\text{Eq. 1})$$

Here $k_D$ denotes the sum of all the donor de-excitation rates excluding FRET. When the fluorophores are confined to two dimensions, interoligomeric proximity FRET can occur between a donor within an oligomer and the distribution of acceptors in different oligomers surrounding it, in addition to the intraoligomeric FRET caused by protein-protein interactions (18). This concept is described mathematically in the kinetic theory formalism by breaking apart $k_{\text{RET}}$ into the sum of two components: $k_{\text{oligo}}^{\text{prox}}$, the rate of donor de-excitation due to FRET between donors and acceptors within an oligomer, and $k_{\text{oligo}}^{\text{inter}}$, the rate of donor de-excitation due to interoligomeric proximity FRET caused by spatial proximity to neighboring acceptors in other oligomers. Thus, we have the following.

$$E_{\text{app}} = \frac{k_{\text{prox}} + k_{\text{oligo}}^{\text{prox}} + k_{\text{oligo}}^{\text{inter}}}{k_D + k_{\text{prox}} + k_{\text{oligo}}^{\text{prox}} + k_{\text{oligo}}^{\text{inter}}} \quad (\text{Eq. 2})$$

As described previously (4), this proximity FRET contribution is a non-negligible component of the total apparent FRET efficiency and is a function of the concentration, interaction propensity, and oligomerization state of the fluorescent protein-tagged membrane proteins.

In an ideal experimental situation, $k_{\text{prox}}^{\text{oligo}} = 0$, and a measurement of $E_{\text{app}}$ would yield a measurement of FRET due to oligomerization as follows.

$$E_{\text{oligo}} = \frac{k_{\text{oligo}}^{\text{prox}}}{k_D + k_{\text{prox}}^{\text{oligo}}} \quad (\text{Eq. 3})$$

This could be achieved practically only if the oligomer concentration may be decreased significantly without breaking the oligomers apart. This will require very strong, specific protein-protein interactions because, according to the law of mass action, low surface density will cause oligomers to dissociate. Such strong lateral interactions are known to occur in the case of multisubunit ion channels, some G-protein-coupled receptors, or other stable oligomers (24, 33–35). However, for many other membrane proteins, the interaction strengths are known to be weak (21, 36, 37).

One could also imagine the opposite situation where only the interoligomeric proximity FRET were measured, as in Equation 4.

$$E_{\text{prox}} = \frac{k_{\text{prox}}^{\text{oligo}}}{k_D + k_{\text{prox}}^{\text{oligo}}} \quad (\text{Eq. 4})$$

Unfortunately, one cannot experimentally decouple the latter contribution from the former, and it is difficult to know when proximity FRET contribution is negligible, especially if the total surface density of labeled membrane proteins is not known. However, by solving for $k_{\text{oligo}}^{\text{prox}}$ in Equation 3 and for $k_{D}$ in Equation 4 and substituting into Equation 2, one can write down the explicit dependence of $E_{\text{app}}$ on $E_{\text{oligo}}$ and $E_{\text{prox}}$ as follows.

$$E_{\text{app}} = \frac{E_{\text{oligo}} + E_{\text{prox}} - 2E_{\text{prox}}E_{\text{oligo}}}{1 - E_{\text{oligo}}E_{\text{prox}}} \quad (\text{Eq. 5})$$

Examination of Equation 5 shows that, if one knows the appropriate proximity FRET contribution, it can be used along with the measured total apparent FRET efficiency to determine the actual FRET due to protein-protein interactions in the membrane. This may be achieved using computer simulations to estimate the interoligomeric proximity FRET (4). Equation 5 can also be rearranged to express the proximity FRET as a function of the total apparent FRET efficiency and the intraoligomeric FRET efficiency.
Examination of Equations 5 or 6 shows that as $E_{\text{oligo}} \to 0$, $E_{\text{prox}}$ as expected for monomeric proteins. Similarly, as $E_{\text{prox}} \to 0$, $E_{\text{app}} \to E_{\text{oligo}}$, showing that if the proximity FRET contribution can be minimized by experimental design, the measured apparent FRET is due to protein-protein interactions.

Sometimes, the two contributions are summed in the literature (i.e. the total apparent FRET efficiency is assumed to be equal to the sum of the oligomeric FRET of protein-protein interactions and the interoligomeric proximity FRET contribution) (27, 38–40). Equation 5 shows that this linear relationship is not correct in the general case. However, if one of the contributions is small, a Taylor expansion yields the following.

$$E_{\text{app}} \approx E_{\text{oligo}} + E_{\text{prox}} \quad \text{(Eq. 7)}$$

Below, we discuss the cases of constitutive monomers, dimers, and monomers in equilibrium, constitutive dimers, and constitutive tetramers with an intrinsic FRET value of 0.70. The same cases, but with a low intrinsic FRET value of 0.30, as well as the additional cases of constitutive trimers and monomer-tetramer equilibrium, are included in the supplemental Results.

Monomers—Fig. 1 (A–C) shows the total apparent FRET efficiency predictions, $E_{\text{app}}$, for the monomer-only simulations. We created two-dimensional configurations of monomeric fluorescent proteins (Fig. 1A), and we simulated $E_{\text{app}}$ using Equations 8 and 9 (see “Experimental Procedures”). In this case, $E_{\text{app}}$ is equal to $E_{\text{prox}}$ because there are no interactions, as shown by Equation 5 when $E_{\text{oligo}} = 0$. Fig. 1B shows the dependence of $E_{\text{app}}$ on the acceptor fraction, $x_A$, for several different total surface densities of labeled receptors. The dashed lines connect data points with equal total fluorophore concentrations. $E_{\text{app}}$ is plotted in Fig. 1C as a function of the total concentration, $[T]$. The dashed lines connect data points with constant acceptor fraction, $x_A$, as indicated on the right.

As shown in Fig. 1, high total apparent FRET efficiencies can be recorded for monomeric receptors, especially at high acceptor concentrations. In cases when the association state is unknown, researchers may blindly apply the two oligomerization models, the Veatch and Stryer model (Equation 18) and the thermodynamic model based on the kinetic theory of FRET (see Equation 27), to interpret the monomeric FRET data. The results of such an analysis of monomeric membrane protein FRET with the two models are shown in Fig. 2.

In Fig. 2A, the simulated total apparent FRET efficiencies are plotted as a function of the acceptor/donor ratio for three different total concentrations of receptors (rec): a very low surface density at $[T] = 1 \times 10^{-5}$ rec/um², a moderate surface density at $[T] = 4 \times 10^{-3}$ rec/um², and a relatively high surface density at $[T] = 8 \times 10^{-3}$ rec/um². We generated 500 data points with random acceptor/donor ratios for each total conc-

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**FIGURE 1. Predictions of the monomer-only simulation.** A, Sample configuration of monomers from the simulation. The figure is drawn to scale, and units on axes are nm. B, $E_{\text{app}}$ plotted as a function of acceptor fraction. The dashed lines connect data points with constant $[T]$, as indicated by the numbers on the right. C, $E_{\text{app}}$ is plotted as a function of the total concentration, $[T]$. The dashed lines connect data points with constant acceptor fraction, $x_A$, as indicated on the right.

The abbreviations used are: rec, receptors; A/D, acceptor/donor; MSE, mean squared error.
FRET Signatures of Membrane Proteins

**FIGURE 2. Analysis results for the monomer-only simulations.** A, the total apparent FRET efficiency, $E_{\text{app}}$, with added random noise as a function of the A/D ratio at $[T] = 8 \times 10^{-5} \text{rec}/\mu\text{m}^2$ (green), $[T] = 4 \times 10^{-3} \text{rec}/\mu\text{m}^2$ (yellow), and $[T] = 1 \times 10^{-1} \text{rec}/\mu\text{m}^2$ (blue). In addition, the best fits of $E_{\text{app}}$ with the model of Veatch and Stryer are shown in red. B, best fit reduced $\chi^2$ versus oligomer order for the analysis of $E_{\text{app}}$ in the presence of random noise with the thermodynamic model based on the kinetic theory formalism, with and without an interoligomeric proximity FRET contribution (green and black symbols, respectively). The dotted line is the 95% confidence cut-off for the reduced $\chi^2$ test: all fits with reduced $\chi^2$ values above this line are rejected, and all fits with reduced $\chi^2$ values below this line are accepted as equally likely models, given the data and their associated error. Analysis without a proximity FRET contribution does produce acceptable models for $n = 1$ and $n = 3$. Analysis with the proximity FRET consideration shows that all models provide an acceptable fit to the data, but the best fit $\Delta G^\circ$ indicates a lack of interaction, and Gaussian-distributed random noise was added to the total apparent FRET efficiency data.

We analyzed the data shown in Fig. 2A with the Veatch and Stryer model, where we optimize for $E_{\text{max}}$ and $n$ (see Equation 18). The best fit parameters are given in Table 1, and the three fits are shown as red lines in Fig. 2A. At all but the lowest surface density, the model falsely indicates the presence of dimers. Of note, at the low surface density fitting, the adjustable parameter $E_{\text{max}}$ is equal to zero, due to a lack of any FRET. In any case, the model of Veatch and Stryer does not correctly predict that the proteins are monomeric because the effect of the proximity FRET, the only contribution to FRET in the monomeric case, is not taken into account in the model. Thus, the use of this model to analyze the total apparent FRET efficiency of monomeric membrane proteins will lead to the incorrect conclusion that the measured FRET occurs due to the presence of dimers.

Next, we again simulated total apparent FRET efficiency arising from monomeric membrane proteins, generating 2,700 data points at random total concentrations, and added Gaussian-distributed random noise to each data point. Fig. 2C shows the simulated total apparent FRET efficiency data as a function of acceptor concentration. We then utilized the kinetic theory formalism to analyze the total apparent FRET efficiency data. The reduced $\chi^2$ value was minimized by optimizing for $E$ and $K_A$ for all models from monomer-only through constitutive hexamer formation, $n = 1:6$ (see Equation 27). For the special case of the monomer-only model, we optimize for the best fit distance of closest approach, $L$, which is twice the exclusion radius for fluorescent proteins (1.4 nm in this work) (see Equation 20).

The best fit reduced $\chi^2$ values are plotted as a function of theoretical oligomer order in Fig. 2B, as black crosses for analysis without the interoligomeric proximity FRET consideration and as green crosses for analysis that includes a numerically estimated proximity FRET contribution. Note that the black and green crosses exactly overlap for $n = 1$ because the same equation (Equation 20) is used in both cases. The overall best fit parameters are given in Table 1. Fitting the total apparent FRET efficiency provides a global minimum for $n = 1$ when interoligomeric proximity FRET is not considered. The reduced $\chi^2$ value for $n = 2:6$, when proximity FRET is taken into account, is low and is indistinguishable from that found for the case of $n = 1$ (Fig. 2B, green crosses). However, the best fit thermodynamic parameters for the oligomeric model fitting indicate a practical lack of interactions with an oligomeric fraction at $\sim 0\%$. All of the models ($n = 1:6$) for the data set with added random error pass the reduced $\chi^2$ test at 95% confidence, indicating that all models represent the data equally well. Because all models indicate a lack of interactions, we conclude that the analysis based on the kinetic theory of FRET yields the correct conclusion when interoligomeric proximity FRET is taken into account.
account for proximity FRET (i.e. if we disregard the analysis for $n = 1$ and we disregard the green crosses in Fig. 2B), it will appear that the reduced $\chi^2$ is minimized at $n = 3$. Thus, we will reach the incorrect conclusion that we have trimers when in fact we have monomers. Failure to account for interoligomeric FRET will therefore lead to erroneous data interpretation with the kinetic theory formalism described here.

We note that whereas it is easy to make a mistake by not accounting for proximity FRET (as demonstrated above), it is also possible to correctly account for it and to arrive at the correct conclusion. When plotted as a function of the acceptor concentration (Fig. 2C), the total apparent FRET efficiency collapses into a curve that is independent of the total concentration and depends only on the acceptor surface densities, in

| Order | Simulation Values | Veatch & Stryer | Kinetic Theory of FRET |
|-------|-------------------|-----------------|-----------------------|
|       | [T] = 1 x 10^{+1} rec/μm$^2$ | [T] = 4 x 10^{+3} rec/μm$^2$ | [T] = 8 x 10^{+3} rec/μm$^2$ |
| 1     | Monomer-only $r_{excl} = 1.4$nm | $n = 3.8$ | $n = 2.2$ | $n = 2.4$ | $\chi^2_{red}$ Minimization (- Proximity FRET Contribution) | $\chi^2_{red}$ Minimization (+ Proximity FRET Contribution) |
|       | $E_{max} = 0.00$ | $E_{max} = 0.29$ | $E_{max} = 0.49$ | Monomer-only: no interactions | All n; no interactions |
| 2     | Constitutive Dimer | $n = 2.0$ | $n = 2.5$ | $> 70\%$ Dimeric Fraction | Constitutive Dimer |
|       | $\bar{E}_{simulated} = 0.70$ | $E_{max} = 0.70$ | $E_{max} = 0.74$ | $\bar{E}_{best \ fit, n=2} = 0.81 \pm 0.01$ | $\bar{E}_{best \ fit, n=2} = 0.70 \pm 0.01$ |
| 3     | Constitutive Trimer | $n = 2.7$ | $n = 3.0$ | $> 90\%$ Oligomeric Fraction | Constitutive Oligomer |
|       | $\bar{E}_{simulated} = 0.70$ | $E_{max} = 0.81$ | $E_{max} = 0.82$ | $\bar{E}_{best \ fit, n=3} = 0.74 \pm 0.01$ | $\bar{E}_{best \ fit, n=3} = 0.70 \pm 0.01$ |
| 4     | Constitutive Tetramer | $n = 2.9$ | $n = 3.1$ | Constitutive Oligomer | Constitutive Oligomer |
|       | $\bar{E}_{simulated} = 0.70$ | $E_{max} = 0.81$ | $E_{max} = 0.82$ | $\bar{E}_{best \ fit, n=4} = 0.61 \pm 0.01$ | $\bar{E}_{best \ fit, n=4} = 0.60 \pm 0.01$ |
accordance with the accepted theory for monomeric proteins (4, 18). As discussed above, we can fit a monomer-only model (Equation 20) and optimize for the distance of closest approach between fluorophores. The fit is excellent and yields $r_{\text{exclusion}} = 1.4 \pm 0.1$ nm (plotted as a red line in Fig. 2C). This is exactly the simulated exclusion radius and is a value that is generally consistent with the size of a fluorescent protein.

The above analysis shows that knowledge of interoligomeric proximity FRET is critically important for the correct interpretation of static quenching FRET measurements of monomeric membrane proteins. Concentrations of donors and acceptors need to be measured, along with the FRET efficiencies, so that the appropriate proximity FRET contribution can be included in the model for the total apparent FRET efficiency. The measured FRET should be plotted as a function of acceptor concentration, and the data should be fitted with Equation 20. Strong sequence-specific interactions will yield values of $r_{\text{exclusion}}$ that are either very small or negative. If, however, $r_{\text{exclusion}}$ is a reasonable measure of the size of the exclusion radius of the protein, then it is highly likely that the FRET measured in the experiment is due to proximity FRET occurring between monomeric proteins.

**Monomer-Dimer Equilibrium**—Fig. 3 and supplemental Fig. S1 show the results of the monomer-dimer equilibrium simulations for a moderate interaction strength with a Gibbs free energy $\Delta G^0 = -4$ kcal/mol. Accordingly, the dimeric fraction ranges from ~0% at the lowest simulated fluorophore density of $1 \times 10^{-1} \text{rec/\mu m}^2$ to a maximum of ~80% at the highest surface densities of $8 \times 10^{-3} \text{rec/\mu m}^2$, as shown in Fig. 3A. The acceptor fraction, $x_A$, was varied from 0.1 to 0.9 at each value of the total concentration in these simulations. With $R_0 = 5.5$ nm, the Förster radius of the mTurquoise-YFP FRET pair, donor-acceptor distances in the simulated dimers were 4.3 and 6.7 nm, corresponding to intrinsic FRET values of $E_\text{intra} = 0.70$ and $E_\text{intra} = 0.30$, respectively. Fig. 3B shows a sample configuration of monomers and dimers, as used in the simulations. A number of acceptors can be seen that are closer to donors than the donor-acceptor distance in the dimer. Fig. 3C shows the total apparent FRET efficiency and the intraoligomeric (dimeric) FRET efficiency as a function of total fluorophore concentration. The oligomeric FRET contribution increases proportionally to the dimeric fraction, and the total apparent FRET efficiency is substantially higher than the intraoligomeric FRET for most of the total concentration range. Fig. 3D shows $E_{\text{app}}$ and the interoligomeric proximity FRET, $E_{\text{prox}}$, plotted as a function of total concentration. The proximity FRET contribution comprises a major component of the total apparent FRET efficiency with a maximum of nearly 50% at $[T] = 8 \times 10^{-3} \text{rec/\mu m}^2$ and $x_A =$
A comparison of the proximity FRET and the intraoligomeric FRET contributions shows that they are approximately of equal magnitude at the highest total surface densities. For the monomer-dimer simulation with $\hat{E} = 0.30$, the stochastic FRET contribution is even greater than the oligomeric FRET contribution to the total apparent FRET efficiency, as shown in supplemental Fig. S1.

In Fig. 4A, the total apparent FRET efficiency and the intrasurface density FRET efficiency are shown as a function of the acceptor fraction, $x_A$, for $[T] = 1 \times 10^{-3} \text{rec/} \mu\text{m}^2$ (bottom curve), $[T] = 4 \times 10^{-3} \text{rec/} \mu\text{m}^2$ (middle curve), and $[T] = 8 \times 10^{-3} \text{rec/} \mu\text{m}^2$ (top curve). $E_{\text{app}}$ is plotted as black circles, connected by black dashed lines, and $E_{\text{oligo}}$ is plotted as blue circles connected by blue dashed lines.

As with the monomeric simulations, we again generated 1,500 data points at fixed total concentrations and random acceptor/donor ratios. Gaussian-distributed random noise was added to the simulated total apparent FRET efficiency values. Fig. 4B depicts the results of the analysis of the monomer-dimer equilibrium data with the model of Veatch and Stryer (see Equation 18). For this analysis, we again fit $E_{\text{app}}$ versus acceptor/donor (A/D) ratio for three values of the total concentration: low (blue), moderate (yellow), and high (green) total receptor concentrations as a function of A/D ratio (red lines) for $\hat{E} = 0.70$ (see supplemental Fig. 1C for $\hat{E} = 0.30$ results). When fitting with the Veatch and Stryer model at the lowest total simulated surface density, the best fit oligomer order is a dimer, $n = 2$, but $E_{\text{max}} = 0.01$ occurs because only proximity FRET was measured, as the dimeric fraction is ~0. For moderate total concentration fitting, we find that the best fit oligomer order is $n = 2.3$, which properly indicates dimer formation with minimal effect by proximity FRET on the fit results. However, in this case, we obtain $E_{\text{max}} = 0.56$, which is not the proper value for $\hat{E}$. When fitting the highest total surface densities, $[T] = 8 \times 10^{-3} \text{rec/} \mu\text{m}^2$, we obtain $E_{\text{max}} = 0.66$, which is close to the simulated value, $\hat{E} = 0.70$. The interoligomeric proximity FRET contribution at this surface density is of the same magnitude as the
intraoligomeric FRET contribution to the total measured apparent FRET efficiency, and the additional curvature gives a best fit \( n = 2.5 \), which could incorrectly be interpreted as a trimer. These results are summarized in Table 2 and in supplemental Table 2 for \( E = 0.30 \). For this low value of intrinsic FRET, we see a very similar behavior in the Veatch and Stryer analysis at low, medium, and high surface densities. Again, dimerization is indicated at low concentration, but at high concentration, the model indicates trimer formation. In both cases of low and high intrinsic FRET, the best fit parameters give no information about the presence of a concentration-dependent monomer-dimer equilibrium. Furthermore, the seeming dependence of \( n \) on the total concentration (due to the stochastic FRET contribution and the concentration-dependent association) could be interpreted as a concentration-dependent change in oligomeric state, with order increasing with total concentration, as we have seen previously.

Fig. 4C shows the results of the reduced \( \chi^2 \) analysis of the monomer-dimer equilibrium simulations with the thermodynamic model based on the kinetic theory of FRET (see Equation 27) for \( n = 1:6 \). The results for the low intrinsic FRET, \( E = 0.30 \), are summarized in supplemental Fig. S2. For the \( E = 0.70 \) simulations, fitting the \( E_{\text{app}} \) versus total concentration data without the numerically estimated interoligomeric proximity FRET contribution leads to a best fit at \( n = 3 \), with \( n = 2 \) and \( n = 3 \) providing similar best fit reduced \( \chi^2 \) values. The best fit of a trimer, when directly fitting the total apparent FRET efficiency, is incorrect and is similar to the results of the Veatch and Stryer model for fitting at high \( T \), where the stochastic FRET contribution is maximal. If one neglects to fit for higher order oligomer formation and only looks for dimers, then the best fit donor-acceptor FRET efficiency in the dimer pair gives \( E = 0.96 \), a non-physical value because of the size of the fluorescent proteins (see Table 2). The reduced \( \chi^2 \) test shows that none of the models for the total apparent FRET efficiency that do not include a proximity FRET contribution produce acceptable fits, given the data and their associated error. With the appropriate proximity FRET correction, the minimum reduced \( \chi^2 \) becomes very pronounced at \( n = 2 \) (see Fig. 4C and Table 2 for the best fit parameters). Furthermore, the \( n = 2 \) model with a proximity FRET contribution is the only model that passes the reduced \( \chi^2 \) test at 95% confidence. Thus, we find that the best fit model is a

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**TABLE 2**

Analysis results of simulations for monomer-dimer (order = 1–2) and monomer-tetramer (order = 1–4) equilibria with the models of Veatch and Stryer and the thermodynamic model based on the kinetic theory of FRET, for \( E = 0.70 \).

The analysis with the model of Veatch and Stryer was performed at three different total concentrations, \([T] = 1 \times 10^{-1} \text{rec/\mu}m^2\), \([T] = 4 \times 10^{-3} \text{rec/\mu}m^2\), and \([T] = 8 \times 10^{-5} \text{rec/\mu}m^2\), and the best fit parameters \( n \) and \( E_{\text{max}} \) are given in the columns. The thermodynamic model based on the kinetic theory formalism was used to fit the simulated FRET data based on reduced \( \chi^2 \) minimization. The black and green text indicates analysis results without and with proximity FRET contribution, respectively.

| Order | Simulation Values | Veatch & Stryer | Kinetic Theory of FRET |
|-------|------------------|----------------|-----------------------|
|       | \( \Delta G^*_{\text{simulated}} = 4.0 \text{ kcal/mol} \) | \( n = 1.9 \) | \( \Delta G^*_{\text{best fit, } n=2} = -3.9 \pm 0.1 \text{ kcal/mol} \) |
|       | \( E_{\text{simulated}} = 0.70 \) | \( n = 2.3 \) | \( E_{\text{best fit, } n=2} = 0.95 \pm 0.01 \) |
|       | \( \Delta G^*_{\text{simulated}} = 12.0 \text{ kcal/mol} \) | \( n = -5 \) | \( \Delta G^*_{\text{best fit, } n=4} = -12.0 \pm 0.1 \text{ kcal/mol} \) |
|       | \( E_{\text{simulated}} = 0.70 \) | \( n = 2.9 \) | \( E_{\text{best fit, } n=4} = 0.68 \pm 0.01 \) |
| 1–2   | \( [T] = 1 \times 10^{+1} \text{rec/\mu}m^2 \) | \( n = 2.5 \) | \( \Delta G^*_{\text{best fit, } n=2} = -4.0 \pm 0.1 \text{ kcal/mol} \) |
| 1–4   | \( [T] = 4 \times 10^{+3} \text{rec/\mu}m^2 \) | \( n = 3.1 \) | \( \Delta G^*_{\text{best fit, } n=4} = 0.59 \pm 0.01 \) |
|       | \( [T] = 8 \times 10^{+5} \text{rec/\mu}m^2 \) | \( n = 2.5 \) | \( \Delta G^*_{\text{best fit, } n=4} = 0.59 \pm 0.01 \) |
dimeric state, with proper extraction of ΔG° and ̂E when proximity FRET is properly taken into consideration. Fig. 4D shows the simulated data with added Gaussian noise and the best fit n = 2 model (compare with Fig. 3C; Eapp versus [T]). For a low value of intrinsic FRET, ̂E = 0.30, the reduced χ² test shows acceptable fits for all n > 1, with the overall minimum occurring at n = 2 (see supplemental Fig. S2). Thus, for a monomer-dimer equilibrium with a low value of intrinsic FRET (long D–A distance in the dimer; see Equation 10), a monomer-dimer model will be difficult or impossible to distinguish from other monomer-oligomer states. However, we do see that the overall global minimum is given at n = 2, showing that the global best fit does indeed represent the true simulated oligomer order.

We investigated the applicability of the linear approximation to Eapp as a sum of proximity and dimeric FRET (see Equation 7) to properly model the simulation predictions for the total apparent FRET efficiency. We utilized the proximity FRET contribution, as defined in Equation 6, in a linear sum with the oligomeric FRET for the monomer-dimer simulations described here with ̂E = 0.30 and ̂E = 0.70. Fig. 5 shows Eapp and the linear approximation as a function of total concentration. The area shaded in green in Fig. 5 shows the acceptable (<10% error in Eapp region for the linear approximation. At low concentrations and low acceptor fractions, the linear approximation (Equation 7) models the total apparent FRET relatively well, whereas for high acceptor fractions and moderate surface densities, the linear approximations overestimate the total apparent FRET efficiency. The linear approximation has a wider range of applicability for low intrinsic FRET values, as can be seen by comparing the xA coverage of the shaded regions in Fig. 5. These complications do not occur when fitting with the full model for the total apparent FRET efficiency (Equation 5).

Constitutive Dimers—Fig. 6 shows the Eapp prediction for dimers, when ̂E = 0.70. The total concentrations were varied from 1 × 10⁻¹ receptors/µm² to a maximum surface density of 8 × 10⁻³ receptors/µm², while the acceptor fraction, xA, was varied from 0.1 to 0.9. Fig. 6A shows one example of a random distribution of dimers utilized in the simulation. Fig. 6B shows the predicted Eapp and Eoligo as a function of the total concentration. As expected, the oligomeric FRET, Eoligo, is a constant (for a given xA) across the entire concentration range. On the other hand, Eapp increases as a function of the total concentration, due to the increasing contribution of proximity FRET. In Fig. 6C, we compare the total concentration dependence of the total apparent FRET efficiency, Eapp, with the interoligomeric proximity FRET contribution, Eprox. Eprox increases from zero as a function of total concentration to a maximum of ~40% for xA, a very significant contribution that cannot be neglected. Supplemental Fig. S3 shows the predictions for dimers when ̂E = 0.30. At this low value of intrinsic FRET efficiency, the proximity FRET contribution is a much greater component of the total apparent FRET efficiency. At the highest total concentrations and acceptor fractions, the magnitude of the interoligomeric proximity FRET contribution is greater than the dimeric FRET contribution to the total apparent FRET efficiency.

For a dimer, Eoligo increases in a linear fashion when plotted as a function of the acceptor fraction, xA (see Ref. 38). Often, this linear relationship is used to discern a dimer from a higher order oligomer. Our simulation predicts this linear behavior, as shown in Fig. 7A. Here, Eoligo is plotted as a function of acceptor fraction with blue circles connected by a dashed line. These data are plotted for [T] = 1 × 10⁻¹ rec/µm², where the proximity FRET contribution is close to zero and for [T] = 8 × 10⁻³ rec/µm² with maximal stochastic FRET. The oligomeric FRET from the two different values of total concentration overlap exactly because proximity FRET has no effect on the magnitude of the intraoligomeric dimer FRET.

The measured total apparent FRET efficiency, Eapp, is also plotted in Fig. 7A for low [T] = 1 × 10⁻¹ rec/µm² and high [T] = 8 × 10⁻³ rec/µm² as a function of acceptor fraction with black circles connected by a dashed line. The Eapp curve for low total surface density overlaps exactly the curve of Eoligo versus xA. Thus, at low surface densities, Eoligo = Eapp and Eapp is also linear with the acceptor fraction (overlap of blue and black lines). When [T] = 8 × 10⁻³ rec/µm², however, the proximity FRET contribution to the total apparent FRET efficiency is significant, and in this case, Eapp is not a linear function of xA (top black curve in Fig. 7A). This effect is also seen in the case of ̂E = 0.30, shown in supplemental Fig. S4A. Thus, the interoligomeric proximity FRET contribution introduces curvature in the plot of Eapp versus xA at moderate to high surface densities for dimers. This curvature is not necessarily an indication of higher order oligomer formation.

Fig. 7 (B–D) and supplemental Fig. S4 show the applications of the models of Veatch and Stryer and the kinetic theory formalism with the simplified model of intraoligomeric FRET to the constitutive dimer data sets with added Gaussian-distributed random noise for ̂E = 0.70 and ̂E = 0.30, respectively. In Fig. 7B, we fit the constitutive dimer predictions with the model of Veatch and Stryer (see Equation 18) and optimized for Emax and n, performing the analysis at two different total concentrations: [T] = 8 × 10⁻³ rec/µm² (high total concentration; green) and [T] = 1 × 10⁻¹ rec/µm² (low total concentration; blue). The best fit Veatch and Stryer fitting results are plotted as red curves in Fig. 7B, and the best fit parameters are given in Table 1 (see Table 2 for ̂E = 0.30). When the total concentration is low and the proximity FRET contribution is negligible, the model of Veatch and Stryer yields the correct oligomerization order, n = 2, and the correct Emax = ̂E, in both cases of ̂E = 0.70 and ̂E = 0.30.
Fitting $E_{app}$ at high total concentration, however, yields $n = 2.5$ for $E = 0.70$ and for $E = 0.30$. This value of $n$ would probably be rounded to 3 by researchers, and thus, the model of Veatch and Stryer does not predict correctly the oligomer size for dimers at high surface densities of membrane proteins. Furthermore, the prediction depends on the total concentration and gives the false impression that the oligomer size increases when the total concentration increases. This seeming dependence of $n$ on total concentration is an artifact occurring because the interoligomeric proximity FRET contributions are not taken into account in the model of Veatch and Stryer.

Fig. 7C shows the best fit reduced $\chi^2$ as a function of oligomer order, for analysis with and without a proximity FRET contribution to the simulated data with added random noise for $E = 0.70$. When the data are analyzed without a proximity FRET contribution, the best fit occurs for $n = 2$ (black crosses). However, none of the models without a proximity FRET contribution are able to pass the reduced $\chi^2$ test, indicating poor fits to the data. When the proper proximity FRET contribution is included in the analysis, the minimum reduced $\chi^2$ value for $n = 2$ becomes very pronounced (green crosses in Fig. 7C), and the simulated parameters are properly extracted through the analysis (Table 1). The dimer model is the only model that passes the reduced $\chi^2$ test at a 95% confidence level. The $\chi^2$ test indicates that the total apparent FRET efficiency of a dimer is statistically different from the FRET efficiencies of other oligomerization orders for $E = 0.70$. Fig. 7D shows the simulated total apparent FRET efficiency with added random noise and the best fit dimer model as a function of total concentration (compare with $E_{app}$ versus $[T]$ in Fig. 6B). For the case of a low value of intrinsic FRET, $E = 0.3$, supplemental Fig. S4C shows that acceptable best fit reduced $\chi^2$ values were obtained for all $n = 2.5$. Supplemental Fig. S4D shows the simulated total apparent FRET efficiency and the best fit dimer model for $E = 0.30$. Thus, for low values of intrinsic FRET, it will be difficult or impossible to exclude various oligomerization states as possibilities, given the measured data. We do see, however, that the absolute minimum occurs at the dimer model, and thus the global best fit does indicate the proper oligomerization state of a dimer, even for low intrinsic FRET.

In conclusion, the use of the model of Veatch and Stryer can lead to misleading results in the case of dimers. Additionally, the donor and acceptor concentrations in the FRET experiments need to be known, such that the kinetic theory formalism can be applied with Equation 5. We find that the interoligomeric proximity FRET contribution must be properly accounted for when analyzing the data of a constitutively dimeric system. When the proximity FRET is taken into account, we are able to correctly identify the presence of constitutive dimers and to recover the correct $E$ from the simulated dimeric $E_{app}$ data. When the acceptor surface density is not known, the proper manner to estimate the appropriate proximity FRET contribution is not immediately apparent.
Constitutive Tetramers.—We next simulated the FRET occurring due to constitutive tetramers. The total concentrations utilized in the simulations ranged from $[T] = 8 \times 10^{-3}$ rec/μm² (top curve) and $[T] = 1 \times 10^{-1}$ rec/μm² (bottom curve). Dashed lines connect data points at constant $[T]$. The $E_{\text{app}}$ curves overlap for all $[T]$. $E$, total apparent FRET efficiency with added random noise as a function of the A/D ratio at $[T] = 8 \times 10^{-3}$ rec/μm² (green) and $[T] = 1 \times 10^{-1}$ rec/μm² (blue). In addition, the best fits of $E_{\text{app}}$ with the model of Veatch and Stryer are shown as red lines. C, best fit reduced $\chi^2$ versus oligomer order for the analysis of $E_{\text{app}}$ in the presence of random noise with the thermodynamic model based on the kinetic theory of FRET both with and without an interoligomeric proximity FRET contribution (green and black crosses, respectively). The dotted line is the 95% confidence cut-off for the reduced $\chi^2$ test. Analysis without a proximity FRET contribution does not produce acceptable models for any order. Meanwhile, for analysis with proximity FRET consideration, the $n = 2$ model is the only model that passes the reduced $\chi^2$ test. D, simulated dimer-only data with added random noise (black) and the best fit $n = 2$ model (red) plotted as a function of total concentration.

A comparison of the interoligomeric FRET contribution for tetramers with that of trimers and dimers shows a drastically reduced interoligomeric proximity FRET contribution in the constitutive tetramer simulations for both $E = 0.70$ and $E = 0.30$. Furthermore, due to the reduced proximity FRET contribution, the intraoligomeric FRET and the total apparent FRET efficiency are nearly equal across all concentration ranges for tetramers at $E = 0.70$. Suplemental Fig. S5C shows that for low intrinsic FRET, $E = 0.30$, interoligomeric proximity FRET becomes a significant component of the total apparent FRET efficiency, contributing to a steeper increase of the total apparent FRET efficiency with increasing surface density of receptors, as compared with the $E = 0.70$ case.

Fig. 9 and supplemental Fig. S6D show the analysis results for the constitutive tetramer simulations with the models of Veatch and Stryer, and the thermodynamic model based on the kinetic theory formalism for $E = 0.70$ and $E = 0.30$, respectively. The results of these analyses are summarized in Table 1.
and supplemental Table 1. In Fig. 9A, we plot the total apparent FRET efficiency as a function of acceptor/donor ratio, for two total concentrations: low surface density, \([T] = 1 \times 10^{-3} \text{ rec/\mu m}^2\) (blue), and high surface density, \([T] = 8 \times 10^{-3} \text{ rec/\mu m}^2\) (green). The reduced interoligomeric proximity FRET contribution in the constitutive tetramer case leads to a reduced total concentration dependence on the curvature of \(E_{\text{app}}\) versus \((A/D)\), when compared with the constitutive dimer simulations. Optimizing for \(E_{\text{max}}\) and \(n\) in the model of Veatch and Stryer (Equation 18), we find that for low and high surface densities, the results for \(\tilde{E} = 0.70\) are nearly identical; \(n\) incorrectly indicates trimer formation with values of 2.9 and 3.1, whereas \(E_{\text{max}}\) does not reflect the true intrinsic FRET of a donor-acceptor pair in the tetramer with a value of 0.81 and 0.82, respectively. The best fit Veatch and Stryer models are shown in Fig. 9A as red lines. Of note is the fact that at a low value of the intrinsic FRET, \(\tilde{E} = 0.30\), the model of Veatch and Stryer incorrectly indicates dimer formation with \(n = 2.3\) for low surface density and gives an indication of trimer formation with \(n = 2.6\) at high surface density. This behavior could be incorrectly interpreted as a concentration-dependent oligomer order shift.

Analysis of the total apparent FRET efficiency data with the thermodynamic model based on the kinetic FRET theory reveals an apparent minimum at \(n = 4\) in the reduced \(\chi^2\) versus oligomer order plot (Fig. 9B) when the interoligomeric proximity FRET is ignored. However, when the proximity FRET contribution is included in the model for the total apparent FRET efficiency, we find that the FRET of tetramer formation is indistinguishable from the total apparent FRET efficiency of trimer formation. Both models for \(n = 3\) and \(n = 4\) pass the reduced \(\chi^2\) test at 95% confidence, as shown by the green crosses in Fig. 9B. In Fig. 9C, we plot the best fit tetramer model for the total apparent FRET efficiency data with added random noise for \(\tilde{E} = 0.70\). For a low value of intrinsic FRET, \(\tilde{E} = 0.30\), and when performing analysis that includes the proximity FRET contribution, the \(\chi^2_{\text{red}}\) values for \(n > 2\) are all nearly equal and deemed acceptable fits (see supplemental Fig. S6 for \(\tilde{E} = 0.30\)). This is a consequence of both the single donor-acceptor distance approximation in Equation 19 and the low \(\tilde{E}\) value and is further enhanced due to the non-uniqueness of the total apparent FRET efficiency for higher order oligomer formation.

In Fig. 10, we compare the uniqueness of the total apparent FRET efficiencies of constitutive trimers (\(\tilde{E} = 0.74\)), tetramers (\(\tilde{E} = 0.70\)), and pentamers (\(\tilde{E} = 0.70\)). The total apparent FRET efficiency values are nearly equal across all concentration values, even at \([T] = 1 \times 10^{-1} \text{ rec/\mu m}^2\), where the proximity FRET efficiency is \(\sim 0\). Thus, FRET of higher order oligomerization is not unique. Other information or experimental constraints will be required to distinguish the oligomer order.

Application to Experimental Data—We have previously published experimental FRET data obtained with a truncated version of the ErbB2 (Her2) receptor, lacking a kinase domain, that is probably monomeric in the plasma membrane (4). Here we
reprocess these previously published data to verify the conclusions of the previous work (4).

Fig. 11 shows the results of the analysis of the ErbB2 data set with the model of Veatch and Stryer and the kinetic theory formalism for the total apparent FRET efficiency. The total apparent FRET efficiency is plotted as a function of total concentration for trimers, $E = 0.74$, as triangles and for constitutive tetramers and constitutive pentamers, $E = 0.70$, as squares and stars, respectively. The total apparent FRET efficiency for higher order oligomerization is not unique across a wide range of concentrations.

FIGURE 10. Comparison of the total apparent FRET efficiency for constitutive trimers, tetramers, and pentamers. The total apparent FRET efficiency is plotted as a function of total concentration for trimers, $E = 0.74$, as triangles and for constitutive tetramers and constitutive pentamers, $E = 0.70$, as squares and stars, respectively. The total apparent FRET efficiency for higher order oligomerization is not unique across a wide range of concentrations.

Fig. 11 shows the results of the analysis of the ErbB2 data set with the model of Veatch and Stryer and the kinetic theory formalism for the total apparent FRET efficiency. Fig. 11A shows the total apparent FRET efficiency, binned and plotted as a function of acceptor/donor ratio, along with the best fit Veatch and Stryer prediction. Analysis with the Veatch and Stryer model gives best fit parameters of $E_{\text{max}} = 0.09 \pm 0.05$ and $n = 1.5 \pm 1$. This analysis would lead to the incorrect conclusion that these proteins are dimerizing, just as predicted earlier for the simulations of monomers.

Fig. 11B shows the best fit mean squared error (MSE) (see Equation 29) as a function of oligomer order for the analysis of the ErbB2 total apparent FRET efficiency with the kinetic theory formalism. There is no minimum in the MSE versus order plot when interoligomeric proximity FRET is taken into account because all of the $n = 1:6$ models provide equally good fits to the data. However, the oligomeric fraction is near zero for all $n = 2:6$. In Fig. 11C, the measured total apparent FRET efficiencies are plotted as a function of the measured acceptor concentration, along with the best fit monomeric model. A comparison of these results with the results for the simulated data in Fig. 2 reveals many similarities in the analysis and the conclusions. Thus, our predictions with ideal simulated data can guide experimental efforts to understand the behavior of data when a proximity FRET contribution is included. Thus, the oligomer order is not discernable for this data set, but the presence of dimers is excluded. C, the apparent FRET efficiency with added Gaussian noise (black circles) and the best fit $n = 4$ model (red circles) is plotted as a function of the total concentration.
Discussion

In this work, we have introduced a new theoretical model for the total apparent FRET efficiency in a static quenching FRET experiment, which describes the explicit dependence of the measurement on the intraoligomeric and the interoligomeric proximity FRET efficiencies. Our goal for this work has been to understand the consequences of ignoring proximity FRET in each case of higher order oligomer formation ($n > 1$) and to determine the ability of the Veatch and Stryer model and the thermodynamic model utilizing the kinetic theory formalism to correctly describe the intrinsic FRET efficiency, oligomeric fraction, and oligomer order. We minimized the complications of oligomer geometry by choosing regular polygons, the most ideal geometry for both the simplified form of the kinetic theory of intraoligomeric FRET and the model of Veatch and Stryer. When the oligomeric geometries deviate from the ideal polygon, the assumption of the single donor-acceptor distance in the oligomer found in both models becomes less realistic and will provide additional difficulty in ascertaining the oligomer order of the protein under study. Furthermore, experimental non-idealities, such as fluorescent protein photobleaching, non-stoichiometric labeling efficiency, and measurement noise, will further add to the difficulties in oligomerization state discernment. Thus, we turned to computer simulations to determine the FRET that would be measured in an idealized situation.

The model of Veatch and Stryer is often utilized by researchers performing semiquantitative FRET experiments on membrane proteins, in which the total surface density of labeled membrane proteins is unknown, but the donor to acceptor ratio is known either through experimental design or measurement (31). We find that, in general, if one does not know the oligomer order before the experiment, the model of Veatch and Stryer will give the researcher confusing and usually incorrect oligomer order results. Particularly surprising was the fact that for monomers, the model indicated dimer formation. In the case of a constitutive dimer, the model of Veatch and Stryer provides a correct result if the concentration is kept low to minimize the proximity FRET contribution. However, fitting of the total apparent FRET efficiency at high receptor surface densities leads to an indication of trimer formation by the model, as a result of the non-negligible proximity FRET contribution. If the acceptor concentrations are not known, then it is difficult to make an appropriate proximity FRET correction to the apparent FRET efficiency or to know whether one is in a concentration regime where proximity FRET is minimized. Overall, we find that this model has strong deficiencies for the determination of oligomer size from bulk static quenching FRET experiments.

Ten years ago, the kinetic theory of intraoligomeric FRET was introduced, which accounts for the total protein concentration and the oligomeric architecture (32), along with a combinatorial mixing contribution to account for random labeling in the oligomer in a static quenching FRET experiment (32). We find that the kinetic theory formalism for the total apparent FRET efficiency can provide the researcher with the proper...
FRET Signatures of Membrane Proteins

Experimental Procedures

FRET Simulation Theory

We performed simulations of FRET between membrane proteins labeled with fluorescent proteins by creating discrete two-dimensional distributions of non-overlapping circles, intended to represent the cross-section of the non-negligible volume of the fluorescent protein β barrel structure. As described by Wolber and Hudson (18), for a two-dimensional static quenching FRET experiment containing multiple excited donors, the ratio of the quantum yields of the donor in the presence and absence of acceptors, $Q_{D,A}/Q_D$, can be estimated through computer simulations by generating many different configurations, $M$, of donors and acceptors in a two-dimensional plane. The total relative configurational quantum yield of the $j$th donor, $q_{j, \text{rel}}^{(b)}$, in configuration $\beta$ is calculated as a sum over all of the donor-acceptor distances in the configuration as follows.

$$q_{j, \text{rel}}^{(b)} = \left ( 1 + \sum_{i=1}^{N_0} (R_{ij}/R_0)^6 \right )^{-1} \quad \text{(Eq. 8)}$$

The relative total configurational quantum yield is then averaged over all of the donors ($N_D$) in a configuration and then over $M$ configurations.

$$Q_{DM}/Q_D = \lim_{M \to \infty} \left [ \frac{1}{M} \sum_{j=1}^{N_D} \sum_{i=1}^{N_0} q_{j, \text{rel}}^{(b)} \right ] \quad \text{(Eq. 9)}$$

In Equation 8 above, $R_{ij}$ is the distance from the $i$th acceptor to the $j$th donor, and $R_0$ is the Förster radius, a property of the donor-acceptor FRET pair (9). The Förster radius is the donor-acceptor distance, $R_0$, at which the intrinsic resonance energy transfer efficiency, $\tilde{E}$, is decreased to 50%.

$$\tilde{E} = \frac{1}{1 + R^6/R_0^6} \quad \text{(Eq. 10)}$$

The total apparent FRET efficiency is then defined as follows.

$$E_{\text{app}} = 1 - Q_{DM}/Q_D \quad \text{(Eq. 11)}$$

The total relative configurational quantum yield of the $j$th quenched donor, $q_{j, \text{rel}}^{(b)}$, is a sum over all acceptors, including acceptors that are intraoligomeric and acceptors that are inter-oligomeric and experience FRET with the donor.

To determine the FRET efficiency due to oligomerization ($E_{\text{oligo}}$ in Equation 3) without a stochastic FRET contribution, we define the oligomeric relative configurational quantum yield of the $j$th donor, $q_{j, \text{rel, oligo}}^{(b)}$ in configuration $\beta$ as a sum over the $i = m, n, o, \ldots$ intraoligomeric acceptor distances to the $j$th donor.

$$q_{j, \text{rel, oligo}}^{(b)} = \left ( 1 + \sum_{i=m,n,o,\ldots}^{N_0} (R_{ij}/R_0)^6 \right )^{-1} \quad \text{(Eq. 12)}$$

In Equation 12, if a donor is monomeric or if an oligomer is composed solely of donors, then $q_{j, \text{rel, oligo}}^{(b)} = 1$. In the case of a donor dimerized with an acceptor, it is equal to $1 - \tilde{E}$, the intrinsic FRET efficiency of the donor-acceptor pair separated by distance $R$ in the dimer, given by Equation 10.

Averaging $q_{j, \text{rel, oligo}}^{(b)}$ over many donors, $N_D$, and many configurations, $M$, leads to the definition of the oligomeric donor-quenched FRET efficiency.

$$Q_{DM, \text{oligo}}/Q_D = \lim_{M \to \infty} \left [ \frac{1}{M} \sum_{j=1}^{N_D} \sum_{i=1}^{N_0} q_{j, \text{rel, oligo}}^{(b)} \right ] \quad \text{(Eq. 13)}$$

$$E_{\text{oligo}} = 1 - Q_{DM, \text{oligo}}/Q_D \quad \text{(Eq. 14)}$$

Similarly to the oligomeric relative configurational quantum yield, the stochastic relative configurational quantum yield of oligomer order, association energetics, and intrinsic FRET efficiency for dimers and mixtures of monomers and dimers. The monomer-only simulation results highlight the importance of examining all known models for their fits to the data. In addition, in the dimer case, the magnitude of the interoligomeric FRET contribution is significant and cannot be ignored in the analysis. Thus, in the case of a monomer-dimer equilibrium or a constitutive dimer, a numeric model for the proximity FRET is necessary to determine the proper association constant and intrinsic FRET efficiency value (donor-acceptor distance in the dimer). Unfortunately, the proximity FRET contribution is only available through the use of computer simulation, but fortunately, it is not difficult to estimate with a computer (see “Experimental Procedures”). For dimers, we find that with intrinsic FRET values <0.5 (donor-acceptor distances greater than $R_0$), dimer formation will be difficult to discern from higher order oligomer models.

With higher order constitutive oligomer formation, $n > 3$, the analysis of the total apparent FRET efficiency with and without a proximity FRET contribution yielded essentially the same results, indicating that the proximity FRET contribution is negligible with respect to analysis for these situations. Interestingly, although the oligomer order was not discernable for $n > 2$, the best fit oligomeric fraction determined for each oligomer order $>2$ closely matched the simulated oligomeric fraction for low and high values of intrinsic FRET in the monomer-oligomer equilibrium simulations. By comparing the total apparent FRET efficiencies for constitutive trimers, tetramers, and pentamers (Fig. 10), it is clear that the total apparent FRET efficiency of higher order oligomer formation is not a unique function of total concentration across all simulated surface densities. In some special cases, complementary structural information may be obtained from other fluorescence methods (6, 7, 24, 28, 35, 41) or from solved crystal structures (42) that can be used in conjunction with FRET measurements to study higher order oligomerization. We have shown that bulk (i.e., average-based), two-color static quenching FRET experiments provide the most useful information in the cases of monomers, equilibrium between monomers and dimers, and constitutive dimers, whereas they are intrinsically limited in their ability to distinguish the order of higher order oligomers, unless additional structural information is available, as discussed above. Nevertheless, we note that although the oligomer order is not distinguishable in this case, the ability to discern non-constitutive oligomer formation from a constitutive oligomeric state remains and can provide new insights into the properties of the membrane proteins engaging in oligomerizing lateral interactions (see supplemental Figs. S11–S14).
FRET Signatures of Membrane Proteins

the jth donor, \( q_{r,j,\text{prox}}^{(b)} \), is defined as the sum over all acceptor distances that do not belong to the same oligomer as the jth donor.

\[
q_{r,j,\text{prox}}^{(b)} = \left( 1 + \sum_{j=1; r \neq m,n,o, \ldots}^{N_0} \left( R_j / R_0 \right)^k \right)^{-1} \tag{Eq. 15}
\]

Next, in the spirit of Equation 5, we return to Equation 8, and we see that the total relative configurational quantum yield can be written in terms of the relative configurational oligomeric and stochastic quantum yields, as shown.

\[
q_{r,j,\text{tot}}^{(b)} = \frac{1}{q_{r,j,\text{prox}}^{(b)} + 1/q_{r,j,\text{oligo}}^{(b)}} - 1 = q_{r,j,\text{prox}}^{(b)} q_{r,j,\text{oligo}} \times \left( q_{r,j,\text{oligo}}^{(b)} \right)^{-1} + q_{r,j,\text{prox}}^{(b)} q_{r,j,\text{oligo}}^{(b)} \times \left( q_{r,j,\text{oligo}}^{(b)} \right)^{-1} \tag{Eq. 16}
\]

Substitution of Equation 16 into Equation 9 shows the complicated dependence of the total apparent FRET efficiency on the stochastic and intraoligomeric relative configurational quantum yields.

\[
E_{\text{app}} = 1 - Q_{\text{dim}} / Q_D = 1 - \lim_{M \to \infty} \left[ \frac{1}{M} \sum_{M=1}^{N_D} \sum_{j=1}^{N_0} q_{r,j,\text{prox}}^{(b)} q_{r,j,\text{oligo}} \times \left( q_{r,j,\text{oligo}}^{(b)} \right)^{-1} \right] \tag{Eq. 17}
\]

**Simulation Protocol**

In this work, the fluorescent proteins are modeled as circles in two dimensions with a 1.4-nm “exclusion radius” as described previously (4), which is intended to represent the exclusion volume of the fluorescent proteins existing in a plane that is parallel to the bilayer.

To model the FRET arising from two-dimensional distributions of donor and acceptor fluorescent proteins with non-negligible finite size, we create non-overlapping ensembles of circles representing donors or acceptors organized as monomers or oligomers of order n and calculate the oligomeric and total relative quantum yield per configuration, as described in Equations 12 and 8. We perform M trials for each acceptor fraction, generating new configurations at each trial, and determine the total and oligomeric FRET efficiencies as shown in Equations 9 and 11 and Equations 13 and 14. The total stochastic FRET contribution is algebraically calculated from \( E_{\text{app}} \) and \( E_{\text{oligo}} \) with Equation 6.

Oligomers are constructed as n-sided regular polygons with circles at the vertices, and with a line connecting the circle centers of the oligomer. The side length, or center-to-center distance for the circles representing fluorescent proteins, is chosen based on a distance determined from Förster’s equation for a given FRET efficiency. A monomer is represented as a simple circle, a dimer as two circles connected by a line, a trimers as an equilateral triangle, tetramers as squares, and pentamers as pentagons with circles at the corners, etc. Non-overlapping ensembles of circles representing oligomers are generated by first choosing a random position and orientation for all oligomers (and monomers). We iterate through the list of oligomers, examining it for overlap with any other oligomers. If overlap occurs, then a new random position is chosen for the oligomer of interest until a suitable position is found. We continue in this manner until all monomers and oligomers are placed in a non-overlapping manner. For the simulation predictions shown in Figs. 1, 3, 5, 6, and 8 and supplemental Figs. S1, S3, S5, S7, and S8 we utilize a pool of 4,000 fluorophores, which are randomly placed in a square region sized according to the desired fluorophore concentration, for \( M = 1,000 \) trials per donor/acceptor ratio per simulated total concentration value. The circles are then randomly assigned to be donor or acceptor molecules. To avoid edge effects, only donors that are positioned five Förster radii, 5\( R_0 \), away from the edges of the region are utilized in the calculations of oligomeric, total, and stochastic FRET, as described above. A Förster radius, \( R_0 = 5.5 \) nm, corresponding to that of the mTurquoise-YFP FRET pair with an assumed \( \kappa^2 = 0.75 \) was used for the simulations in this work. The quantum yield of mTurquoise, the donor, is 0.84 (\( Q_D \)), and the quantum yield of the acceptor, YFP, is 0.61 (\( Q_A \)). Simulated surface densities range from a minimum of \( 1 \times 10^{-1} \) rec/\( \mu m^2 \) to a maximum of \( 8 \times 10^{-3} \) rec/\( \mu m^2 \), with acceptor fractions ranging from 0.1 to 0.9 in steps of 0.1. We performed simulations at two values of intrinsic FRET: \( E = 0.70 \), corresponding to a donor-acceptor distance of 4.7 nm, and \( E = 0.30 \), corresponding to a donor-acceptor distance of 6.3 nm.

**The Model of Veatch and Stryer**

In some experimental situations, the concentrations of donors and acceptors may not be known, but the ratio of donor to acceptor molecules is known or measured along with the total apparent FRET efficiency. In this case, the model of Veatch and Stryer is often utilized to determine the oligomeric state of the membrane proteins of interest (31), given by the following.

\[
E_{\text{app}} \approx E_{\max} \left( 1 - x_D^{-1} \right) = E_{\max} \left( 1 - \left( \frac{[A]}{[D]} \right)^{-n-1} \right) \tag{Eq. 18}
\]
which a number of donors and acceptors can be found within an oligomer. Lastly, this model does not account for the presence of interoligomeric proximity FRET. For \( n = 1 \), monomeric proteins, the model predicts zero FRET.

For every case discussed under “Results,” we generated 500 data points, at fixed total surface densities of fluorophores, and we allowed the acceptor fractions to vary randomly from 0 to 1. Gaussian-distributed random error was generated for each data point, \( \mu = 0 \) and \( \sigma = 0.03 \), giving absolute error values typically within 0–9%. This random error was added to the simulated total apparent FRET efficiency to create a simulated data set with noise similar to that found in actual experiments. These data were then fit with Equation 18. The best fit parameters, \( E_{\text{max}} \) and \( n \), are given in Tables 1 and 2 for simulations with \( \tilde{E} = 0.70 \) and in supplemental Tables 1 and 2 for \( \tilde{E} = 0.30 \). These data are shown in Figs. 2A, 4A, 7B, and 9B and supplemental Figs. S2B, S4B, S6A, S9A, and S10A, where the total apparent FRET efficiency is plotted as a function of acceptor/donor ratio. The best fit Veatch and Stryer results are plotted in each of the figures as red lines.

The Kinetic Theory of Intraoligomeric FRET

The kinetic theory formalism was described by Raicu in 2007 (32), where it was applied to develop a theoretical relation for the FRET occurring due to oligomeric assemblies of labeled proteins. The kinetic theory formalism for intraoligomeric FRET provides a theoretical description of the FRET that will be measured for an ensemble of labeled oligomers (32, 43) while excluding proximity FRET. A complete description of the system requires knowledge of the geometries of the donor-acceptor distances within an oligomer, which are included in the full theory. These distances are known in these simulations, but in many real-world experimental situations, these distances are unknown. In some cases, the donor-acceptor distances may be inferred from separate experiments (see “Discussion”). A simplified expression was derived for use in this case where these distances and geometries are not known by assuming equal donor-acceptor distances for all donor-acceptor pairs in the oligomer. In that particular case, the kinetic theory formalism yields the following theoretical expression for the intraoligomeric FRET.

\[
E_{\text{oligo}}^{Dq} = \frac{\mu_{\text{oligo}}}{D} \sum_{k=1}^{n-1} \frac{k(n-k)\tilde{E}}{1 + (n-k-1)\tilde{E}} \binom{n}{k} p_D^k P_A^{n-k} 
\]

(Eq. 19)

In Equation 19, \( n \) represents the number of constituents (or protomers) in an oligomer, or the “oligomer order,” and \( \tilde{E} \) represents the “pairwise FRET efficiency” or “intrinsic FRET” (i.e. the efficiency between a single donor-acceptor pair in the oligomer, chosen as a reference) (see Equation 10). \( \mu_{\text{oligo}} \) represents the concentration of oligomers in the ensemble. \( p_D \) and \( P_A \) are the probabilities of finding donors and acceptors in the oligomer. For large numbers of molecules, the latter two quantities are equal to the fraction of acceptors and donors, respectively: \( x_A = [A]/([D] + [A]) \), \( x_D = 1 - x_A \), with \([D]\) and \([A]\) representing the total donor and acceptor concentrations. It is through \( \mu_{\text{oligo}} \) that the kinetic theory of intraoligomeric FRET can be utilized to determine the thermodynamic properties of membrane proteins by application of the law of mass action, described below.

Equation 19 gives the theoretical description of the intraoligomeric donor-quenched energy transfer efficiency for dimers and higher order oligomers, \( E_{\text{oligo}} \) assuming an equal donor to acceptor distance for all donor-acceptor pairs in the oligomer. For the case of \( n = 2 \), a dimer, and \( n = 3 \), a trimer, arranged as an equilateral triangle, this simplified model for intraoligomeric FRET is always correct because there is only one donor-acceptor distance in the oligomer. For tetramers and above, this is an approximation that minimizes the number of adjustable parameters in the theoretical model for FRET (32).

Building a Thermodynamic Model of Intraoligomeric FRET with the Kinetic Theory

A Special Case, Monomeric Membrane Proteins—If there are no specific interactions between the membrane proteins, only proximity FRET will occur. The apparent FRET efficiency for two-dimensional distributions of fluorescent proteins confined to a plane is described by the numeric results of Wolber and Hudson (18) and King et al. (4) The prediction for the relative quantum yield of a monomeric donor in the presence of random configurations of acceptors as a function of acceptor concentration, \( R_o \), and distance of closest approach, \( L \), is given by the following exponential function,

\[
Q_{\text{DA}}/Q_D = \exp(A(L, R_o) \cdot C) 
\]

(Eq. 20)

where \( A(L, R_o) = \sum_i a_i (L/R_o)^i \), \( C = [A]R_o^2 \) is a dimensionless acceptor concentration, and the values of \( a_i \) are given by King et al. (4). In this work, the exclusion radius is 1.4 nm, giving \( L/R_o = 0.51 \).

Oligomerizing Membrane Proteins—Here we describe the general derivation of \( E_{\text{oligo}} \) for any oligomer of order \( n \). The specific cases of dimers, trimers, tetramers, etc., can be generated by setting \( n = 2, 3, 4 \), etc. For the case of a monomer-oligomer equilibrium, the theoretical intraoligomeric FRET efficiency is described by Equation 19. Letting

\[
E = \sum_{k=1}^{n-1} \left[ \frac{k(n-k)\tilde{E}}{1 + (n-k-1)\tilde{E}} \binom{n}{k} p_D^k P_A^{n-k} \right] 
\]

(Eq. 21)

and multiplying the right side by \([T]/[T]\), we have the following.

\[
E_{\text{oligo}}^{Dq} = \frac{\mu_{\text{oligo}}}{[D]} \left[ \frac{T}{[T]} \right] E = \frac{f_{\text{oligo}}}{n \cdot x_D} \cdot E 
\]

(Eq. 22)

Next, we focus on the fraction of molecules associated into oligomers, \( f_{\text{oligo}} \),

\[
f_{\text{oligo}} = \frac{n \cdot \mu_{\text{oligo}}}{[T]} 
\]

(Eq. 23)

Here, as before, \( \mu_{\text{oligo}} \) is the concentration of oligomeric receptors. For a monomer-oligomer equilibrium, we define an association constant as follows.
**FRET Signatures of Membrane Proteins**

\[
K_n = \frac{\mu_{\text{oligo}}}{[m]^n} \tag{Eq. 24}
\]

Writing the total concentration of macromolecules in terms of \(K_n\) and \([m]\) and rearranging the \(n\)th order polynomial gives the following.

\[
nK_n[m]^n + [m] - [T] = 0 \tag{Eq. 25}
\]

Next, we use a root-finding algorithm to find the largest positive real root, \([m]/(K_n, [T])\), and write the fraction of oligomers as follows.

\[
f_{\text{oligo}} = \frac{n\mu_{\text{oligo}}}{[T]} = \frac{nK_n[m]^n}{[T]} \tag{Eq. 26}
\]

The thermodynamic model of intraoligomeric FRET for a mixed population of monomers and oligomers can then be calculated from the oligomeric fraction, \(f_{\text{oligo}}(K_n, [T])\), according to the following.

\[
E_{\text{Dq}}^{\text{oligo}} = \frac{f_{\text{oligo}}(K_n, [T])}{n \times N} \cdot E \tag{Eq. 27}
\]

In cases when the oligomeric fraction, determined in the fit, exceeds 95% over the very broad range of concentrations used in the simulations (1 \(\times 10^{-1} \) rec/\(\mu\)m\(^2\) through 8 \(\times 10^{-3} \) rec/\(\mu\)m\(^2\)), we term the best fit oligomer a “constitutive oligomer” (see Tables 1 and 2 and Supplemental Tables 1 and 2).

**Analysis of \(E_{\text{app}}\) with a Library of Numeric Proximity FRET Estimations**—The published model for intraoligomeric FRET based on the kinetic theory formalism summarized above does not account for proximity FRET (18). We know from previous work that the proximity FRET contribution depends on the oligomeric fraction and the oligomer order (4). However, its relative contribution to the measured FRET efficiencies as a function of oligomer order is unknown. To investigate this issue, we compare the fitting results with and without a stochastic FRET contribution in Tables 1 and 2 and supplemental Tables 1 and 2.

Because there is no analytic form for the proximity FRET occurring between fluorophores with non-negligible size as a function of acceptor concentration, we simulated proximity FRET for all \(n = 2:6\) over a gridded multidimensional space of the two adjustable parameters, \(\tilde{E}\), and \(K_n\), for acceptor concentrations ranging from zero to 8 \(\times 10^{-3} \) acceptors/\(\mu\)m\(^2\) (see “Simulation Protocol”), with a 1.4-nm exclusion radius. \(\tilde{E}\) values ranged from 0.05 to 0.90, with association constants, \(K_n\), varying from monomer-only (\(K_n \rightarrow 0\)) through constitutive oligomerization (\(K_n \rightarrow \infty\)) in steps of 0.5 kcal/mol. This “library” was used to perform a gridded search for the best fit model to the data set (44) by building a theoretical model for the total apparent FRET efficiency that includes the appropriate numerically estimated interoligomeric FRET contribution and the simplified model for intraoligomeric FRET in the full kinetic theory model for the total apparent FRET efficiency, as given in Equation 5.

The interoligomeric proximity FRET is a function of all of the parameters of interest in a FRET measurement, \(n\), \(\tilde{E}\), and \(K_n\), and accordingly, the choice of a proximity model fixes the choice of the parameters in terms of the fraction of oligomers and the intrinsic FRET.

It is computationally expensive to estimate the proximity FRET for every possible combination of the intrinsic FRET and oligomeric association constant (there are an infinite number of combinations), and furthermore, small changes to these parameters have little effect on the magnitude of the proximity FRET contribution. As such, we first performed the analysis with the library described above, and then we “polished” the best fit intrinsic FRET and oligomeric association constant values by fixing the best fit proximity FRET model to that found above in the gridded search. We utilized a MATLAB non-linear least squares fitting algorithm to vary and find the best fit values of the intrinsic FRET, \(\tilde{E}\), and the equilibrium association constant, \(K_n\), and their 95% confidence intervals (see Equations 5 and 19).

We performed two separate analyses utilizing the kinetic theory formalism: with and without a proximity FRET contribution (see Equations 5 and 19). For every case discussed under “Results,” we generated 2,700 data points with randomly chosen total surface densities and acceptor fractions over the same ranges as before: 0.1–0.9 in steps of 0.1. Gaussian-distributed random error was generated for each data point, \(\mu = 0\) and \(\sigma = 0.08\), giving absolute error values typically within 0–24%. This random error was added to the simulated total apparent FRET efficiency to create a simulated data set with noise similar to that found in actual experiments (4, 37, 38, 45–47).

For all \(n\), we fit the total apparent FRET efficiency, as shown in Figs. 2C, 4D, 7D, and 9D and supplemental Figs. S2D, S4D, S6C, and S9C, directly with the simplified kinetic theory of intraoligomeric FRET (as in Equation 19) and ignore the interoligomeric proximity FRET contributions. These results are summarized as a plot of the best fit reduced \(\chi^2\) value as a function of oligomer order, \(n\), as black crosses. Additionally, for \(n = 2:6\), we utilized a proximity FRET contribution (described above) in the kinetic theory of the total apparent FRET efficiency, Equation 5, and examine the minimized reduced \(\chi^2\) value and best fit parameters of the model as a function of oligomer order. The best fit model utilizing the proximity FRET contribution is also plotted in the same figures above with the simulation predictions. The analysis results when including the proximity FRET contribution are summarized in the reduced \(\chi^2\) versus oligomer order plots with a green cross. The reduced \(\chi^2\) value is calculated for two adjustable parameters according to Equation 28.

\[
\chi^2_{\text{red}}(K_n, \tilde{E}) = \frac{1}{N - 2 - 1} \times \sum_{i=1}^{N \text{ data points}} \frac{(E_{\text{app, theo}} - E_{\text{app, i}})^2}{\sigma_i^2}
\]

\(\tag{Eq. 28}\)

For all \(n = 1:6\), we minimize the reduced \(\chi^2\) value shown in Equation 28 and choose the model with the overall minimum reduced \(\chi^2\) value as the best fit model given the data set and its associated error.

We then subjected the reduced \(\chi^2\) values for the best fit value of every \(n\) to the \(\chi^2\) test for a 1 – \(\alpha\) = 0.95 confidence limit. All models with a reduced \(\chi^2\) value of <1.04 are deemed acceptable.
within the 95% confidence limit. A magenta line representing the 95% cut-off limit is plotted along with the best fit reduced \( \chi^2 \) values. All models with a reduced \( \chi^2 \) value above the line (\( \chi_{\text{red}}^2 \)) are rejected as statistically different from the measured data, whereas all models below the cut-off are accepted. Finally, the MSE is calculated using Equation 29.

\[
MSE(K, \hat{E}) = \frac{1}{N} \times \sum_{i=1}^{N} (E_{\text{app, theo}} - E_{\text{app, app}})^2
\]  

(Eq. 29)

**Author Contributions**—C. K. and V. R. derived the equations. C. K. designed the simulations, performed the data analysis, and prepared the figures. C. K. and K. H. wrote the manuscript.

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