New and Notable

Taking Care of Bystander FRET in a Crowded Cell Membrane Environment

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Oligomerization of membrane proteins is a key event in cell signaling, and yet it is challenging to explore experimentally due to the complexity associated with cell membranes (1). The article by King et al. (2) in this issue of the *Biophysical Journal* describes a strategy for avoiding a major problem in exploring interaction between membrane proteins utilizing fluorescence resonance energy transfer (FRET). The problem has to do with FRET arising from membrane proteins that do not interact, but still give rise to FRET because they happen to be within the required distance for energy transfer (see Fig. 1). This issue assumes relevance in view of the highly crowded nature of the cell membrane (3). FRET from such noninteracting (bystander) pairs complicates the interpretation of FRET results. To our knowledge, King et al. (2) have offered, for the first time, an experimentally verified theoretical framework for membrane proteins, which can be effectively used to correct for bystander FRET.

FRET is a powerful biophysical tool for determining proximity relationships between fluorescently-tagged macromolecules. The photophysical consequences of FRET from an initially excited donor molecule to an acceptor molecule are well understood, and include 1), the quenching of donor emission and donor excited state lifetimes and 2), the increase in sensitized emission from the acceptor and the corresponding kinetics of the sensitized emission. These changes in photophysics can be quantitatively converted into an energy transfer efficiency that is related to the proximity between donor and acceptor probes on the 1–10 nm scale (4). Once an energy transfer efficiency is extracted experimentally, one is faced with the problem of how to interpret the experimental results. For dilute complexes in solution, there are multiple factors that affect measured energy transfer efficiencies.

These are:

1. Spectral overlap between donor emission and acceptor absorption,
2. The orientation between donor and acceptor transition moment dipoles,
3. Stoichiometry,
4. Proportion of fluorophores as free and bound to the complex, and
5. Distance between the donor and acceptor.

For well-characterized systems in solution, some of these factors can be taken into account and reasonable estimates of distances, or indeed relative changes in distance, can be extracted.

For membrane proteins, where cellular expression systems could lead to high levels of proteins at the cell membrane due to lack of control in the expression levels (5), the possibility of FRET occurring from proximal but noninteracting molecules needs to be taken into account (Fig. 1). This is crucial for interpreting FRET in membranes in terms of protein-protein interactions or oligomeric state of membrane proteins. Energy transfer between randomly distributed donors and acceptors in a two-dimensional plane (such as the biological membrane) has been the subject of many theoretical and experimental studies (6–9). However, until now there has been no reliable experimental system for membrane proteins to test the theoretical predictions of these models.

The article by King et al. (2) addresses an important issue in the usage of FRET to determine quaternary structures of membrane proteins. What is the contribution of bystander or proximity to the measured FRET efficiency? The authors achieve this goal through two methods. First, they use simulations of model oligomeric distributions to extract theoretical proximity FRET values as a function of acceptor concentration. The novelty here is the effect of oligomeric state (i.e., dimer, trimer, or tetramer) on proximity FRET, which has not been examined previously. Second, the authors use YFP donor/mCherry acceptor monomeric membrane protein constructs as experimental model systems for examining proximity FRET. The experimental results agree well with the theoretical framework, even allowing determination of distances of closest approach.

The implications for experimental design in future FRET experiments are clear. Expression levels should be kept to a minimum to avoid bystander FRET. According to the experiments of King et al. (2), a 20% bystander FRET efficiency corresponds to an acceptor density of 2000 molecules/μm^2. Given that typical cell surface areas range between 1000 and 5000 μm^2, this density corresponds to an expression level of 2–10 $\times$ 10^6 proteins per cell. Such high levels of expression would lead to complications due to bystander FRET and should be avoided. This article makes a significant contribution to understanding the limitations of FRET-based approaches to membrane protein structure determination, and could serve as a benchmark for exploring organization and interactions of membrane proteins utilizing FRET.

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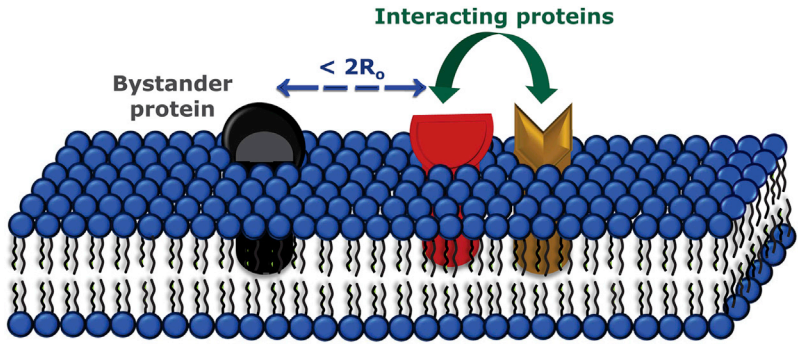

FIGURE 1 A schematic representation of the cellular membrane showing bystander FRET. (*Solid double-headed arrow*) Interacting protein pair. (*Shaded*) Bystander protein, with a distance of separation $<2R_o$ (4). The article by King et al. (2) outlines a strategy to take care of energy transfer from bystanders, thereby increasing the rigor and accuracy of the FRET analysis and interpretation.

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