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New and Notable

A Toolbox of Fluorescence Microscopic Approaches Reveals Dynamics and Assembly of a Membrane-Associated Protein

Andrew H.A. Clayton[‡*]
and Amitabha Chattopadhyay[†*]
[†]Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Hyderabad, India; and
[‡]Centre for Microphotonics, Faculty of Engineering and Industrial Sciences, Swinburne University of Technology, Hawthorn, Victoria, Australia

Aggregation and oligomerization have often been challenging yet exciting aspects in the study of membrane-associated proteins (1). The motivation for exploring oligomerization of membrane proteins derives from the emerging realization that oligomerization holds the key to efficient drug discovery (2). Although oligomerization is an important step for membrane proteins, dynamics play a crucial role in protein function in membranes. Unfortunately, oligomerization studies often do not address the issue of dynamics (and vice versa). In this issue of the *Biophysical Journal*, Crosby et al. (3) describe a comprehensive study in which both assembly and dynamics of a membrane-associated protein, annexin A4, are explored. The elegance of this article lies in arriving at a model of annexin assembly at the plasma membrane, by an intelligent combination of complimentary fluorescence microscopic approaches that could provide useful insight into its function.

A summary of the key membrane-associated processes along with the measurement techniques utilized in the study of Crosby et al. (3) is shown in Fig. 1. Crosby et al. (3) examined annexin transfer from the cytosol to the membrane surface (Fig. 1, membrane binding) by total internal reflection fluorescence microscopy (TIRF). TIRF exploits the exponential decay of the evanescent field near an interface to excite molecules within ~100 nm of the interface. These measurements revealed a time-dependent increase in the number of annexins at the plasma membrane.

Two Förster resonance energy transfer (FRET) approaches were utilized to address the self-assembly of annexin. In the first method (TIRF-FRET-FLIM), FRET was determined by the reduction in excited state lifetime of a donor fluorophore when it transfers energy to an acceptor fluorophore. Fluorescence lifetime imaging microscopy (FLIM) is a robust way to determine energy transfer because it is insensitive to intensity (4), and the permutated recording method previously developed by the Gadella laboratory (5) makes it insensitive to photobleaching artifacts. To increase the time resolution of the FRET measurements, the authors utilized a second approach, i.e., an intensity ratio-based method that relies on the decrease in quantum yield of the donor fluorophore, and increase in the sensitized emission of the acceptor fluorophore. The time resolution of the intensity ratio method is limited only by the integration time of the camera. The authors were able to show robust FRET using TIRF-FRET-FLIM, and the intensity ratio method provided evidence for increase in FRET after annexin binding to the membrane surface. The latter observation is consistent with a bona fide assembly process, as distinct from trivial (random) FRET that could occur due to overexpression. FRET is sensitive to proximity (in the range of 1–10 nm), and is dependent on factors such as orientation factor, local concentration, and stoichiometry of complexes.

Number-and-brightness analysis (6) is a powerful tool to determine the stoichiometry of mobile complexes. This method derives from the family of fluctuation spectroscopy and determines the molecular brightness from the statistical properties (average intensity and variance in intensity) of a fluorescence photon stream with pixel spatial resolution in an image. To measure the assembly state of annexin (Fig. 1, cluster size), the authors compared the brightness of visible fluorescent protein fusions of annexin in the cytosol and membrane, with control constructs of known stoichiometry. The data convincingly revealed monomeric annexin in the cytosol, which formed trimers upon binding at the membrane surface. Importantly, because number-and-brightness analysis is a fluctuation technique, it robustly determines the assembly state of codiffusing molecules. Conversely, it cannot determine the stoichiometry of immobile complexes. This aspect is appreciated by the authors when interpreting their results.

To monitor lateral dynamics, the authors performed fluorescence recovery after photobleaching (FRAP), a commonly used technique for the measurement of membrane protein dynamics (7). The authors analyzed their FRAP data in terms of diffusion, mobile/immobile fractions, and binding/unbinding processes. Significantly, the authors noted the appearance of large aggregates or clusters of annexins in the fluorescence images. The appearance of these clusters was correlated with an increase in immobile fraction in FRAP experiments. The increase in FRET after annexin binding to the surface was attributed to these higher-order immobile aggregates.

Taken together, the authors were able to derive a model that incorporates monomer-to-trimer assembly (number-and-brightness analysis), diffusion of trimers at the membrane (FRAP and number-and-brightness analysis), and higher-order assembly of immobile

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*Correspondence: amit@ccmb.res.in or aclayton@swin.edu.au

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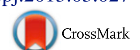

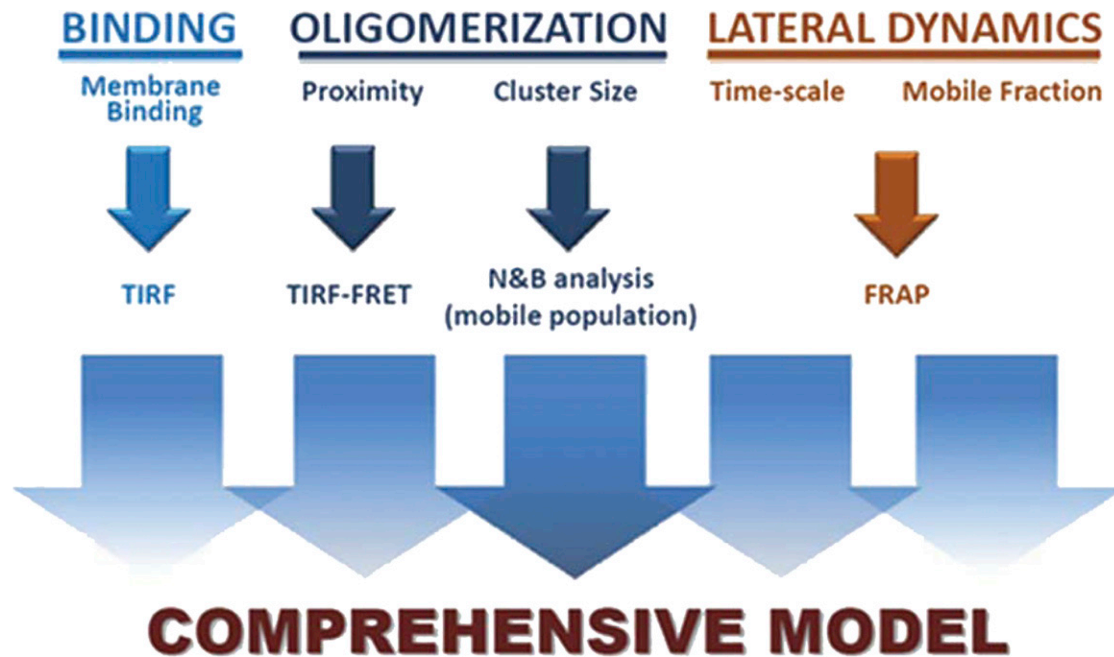

FIGURE 1 A schematic representation of the strategies employed to examine the assembly and dynamics of membrane-associated proteins. (*Top line*) Membrane processes. (*Small arrows*) Techniques used to measure specific membrane processes. An interesting feature is the complementarity of the approaches, which leads to a comprehensive understanding.

complexes (FRET and FRAP). This model provides a generalization of previous studies that were limited in scope. Importantly, we believe the article by Crosby et al. (3) represents a paradigm shift in the way membrane-associated macromolecular assembly and dynamics are measured. The majority of contemporary drug targets are membrane proteins (8), and display complex oligomerization (monomer/oligomer/cluster) and dynamics (non-Brownian diffusion). Despite impressive advances in membrane protein crystallography in the last few years (9), a robust understanding of membrane protein function requires knowledge of dynamics and assembly. A judicious combination of biophysical approaches such as the ones used by Crosby et al. (3) would provide comprehensive information about these processes. Such information on membrane proteins would allow better and more effective therapeutic approaches.

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