

Review

Fluorescence Approaches for Determining Protein Conformations, Interactions and Mechanisms at Membranes

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Processes that occur at membranes are essential for the viability of every cell, but such processes are the least well understood at the molecular level. The complex nature and physical properties of the molecular components involved, as well as the requirement for two separated aqueous compartments, restrict the experimental approaches that can be successfully applied to examine the structure, conformational changes and interactions of the membrane-bound proteins that accomplish these processes. In particular, to accurately elucidate the molecular mechanisms that effect and regulate such processes, one must use experimental approaches that do not disrupt the structural integrity or functionality of the protein-membrane complexes being examined. To best accomplish this goal, especially when large multicomponent complexes and native membranes are involved, the optimal experimental approach to use is most often fluorescence spectroscopy. Using multiple independent fluorescence techniques, one can determine structural information in real time and in intact membranes under native conditions that cannot be obtained by crystallography, electron microscopy and NMR techniques, among others. Furthermore, fluorescence techniques provide a comprehensive range of information, from kinetic to thermodynamic, about the assembly, structure, function and regulation of membrane-bound proteins and complexes. This article describes the use of various fluorescence techniques to characterize different aspects of proteins bound to or embedded in membranes.

Key words: cholesterol-dependent cytolysin (CDC), conformational changes, fluorescence resonance energy transfer (FRET), fluorescence spectroscopy, membrane protein structure, membrane, nascent protein chains, perfringolysin O (PFO), protein, protein-membrane interactions

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Proteins interact with membranes in a multitude of ways. Some are embedded, often by co-translational integration during their synthesis by ribosomes, in the nonpolar core of the phospholipid bilayer to form integral membrane proteins. Some are bound to the surface of the membrane by mechanisms ranging from noncovalent association with a membrane component(s) to covalent modification that attaches to the protein a nonpolar moiety that mediates membrane binding (e.g. palmitoylation). Such proteins, termed peripheral membrane proteins, may bind to the surface either reversibly or irreversibly. In some cases, the nature of a protein's interaction with the membrane may vary depending upon the circumstances. For example, some proteins are soluble and stable in aqueous solution until they are exposed to a membrane surface that triggers their binding to, and sometimes insertion into, the bilayer. Proteins involved in blood coagulation or apoptosis bind to cell surfaces to effect clot formation or cell death only after cell activation elicits the exposure of negatively charged phosphatidylserine on the cell surface (1,2). Similarly, perfringolysin O (PFO), a member of the cholesterol-dependent cytolysin (CDC) family of bacterial protein toxins (3,4), is soluble in aqueous solution even at concentrations exceeding 10 mg/mL (5), but binds to mammalian membranes containing cholesterol and forms a large hole, thereby killing the cell (3,4).

Yet, this wide variety of protein interactions with membranes is not the reason that the study and understanding of membrane proteins has lagged far behind those of soluble proteins. Instead, the experimental characterization of proteins bound to or embedded in a membrane has progressed much more slowly because examining such proteins under native conditions requires the study of a two-phase system that typically consists, at a minimum, of a nonpolar milieu that separates two distinct aqueous compartments. Such a complex biochemical system is incompatible with many experimental approaches, most notably with X-ray crystallography, cryoelectron microscopy (cryoEM) and NMR, three techniques widely perceived to be definitive in terms of determining the structure of macromolecules and macromolecular complexes.

As three-dimensional crystals of integral membrane proteins cannot be formed in the presence of a phospholipid bilayer, membrane proteins are crystallized out of solutions containing detergent molecules that substitute for the nonpolar environment in the core of the bilayer. Thus, the atomic-resolution crystal structure of a membrane protein may be distorted from its native structure because of the absence of the lipid materials that normally surround the protein's hydrophobic transmembrane segments (TMSs) and thereby influence protein conformation. Similarly, to obtain high-resolution membrane protein structures from image reconstructions of single particle cryoEM data, one must eliminate the membrane. These techniques are therefore not ideal for studying membrane protein structure because the polypeptides are not in their biphasic native milieu surrounded by both lipids and water. NMR cannot currently be used to solve the structure of large proteins, and the high protein concentrations generally required for NMR analysis are very difficult to achieve with proteins embedded in or bound to phospholipid vesicles. It is also important to note that the functionality of the crystal and cryoEM structures cannot be assayed because the data are obtained from nonfunctional samples under non-native conditions. As a result, one should be cautious in interpreting crystal and cryoEM data, especially those derived from membrane proteins.

In stark contrast, many spectroscopic techniques can examine membrane protein samples under native conditions. Of these, fluorescence spectroscopy is the most useful and versatile, for the reasons noted below. The environment of a specific site on a protein can be determined and monitored under conditions that permit the simultaneous assessment of system functionality, as well as any changes that occur as a function of time. By correlating spectral data with specific structural and functional states, an investigator can provide direct and often unambiguous information about the structures, interactions and mechanisms by which a membrane protein accomplishes its tasks.

One recent example illustrates the value of fluorescence-based studies of membrane proteins. Cryoelectron microscopy images of membrane-bound pneumolysin were used to generate a detailed model of CDC structure and interactions with the membrane in 1999 (6). In 2005, a model that differed markedly from the previous one was presented by the same group (7). The dramatic change in interpretation of the EM images occurred because of a series of papers that used fluorescence to characterize the stages of pore formation by the PFO CDC, from the identity and conformation of the sequences that form the hole in the bilayer (8,9) to the topography of individual PFO domains relative to the membrane surface at different stages of pore formation (10,11) to the mechanism of pore formation (5,12–16). Thus, guided by this detailed and comprehensive knowledge gained from the published fluorescence experiments, the cryoEM images and

mechanisms were re-interpreted and now correspond precisely with the structures and mechanisms determined previously using fluorescence.

In this article, I will first discuss a few aspects of preparing and using fluorescent-labeled proteins. A series of frequently asked questions about membrane protein structure will then be presented, and the fluorescence technique(s) that can address each will be described. Although the brevity of this paper restricts the number of examples that can be discussed and cited below (hence, most are from our own studies), the techniques described can be applied to all types of proteins that associate with or insert into a membrane. Also, because of space limitations, many practical details and cautions about procedures and analyses that are critical for obtaining and interpreting different spectral data properly have not been included, but I have included as many as possible. Readers are therefore encouraged to consult more comprehensive sources as needed (e.g. 17,18).

Fluorescence: What, Why and How?

What?

Fluorescence is a phenomenon with two distinct stages, excitation and emission. A fluorescent chromophore (fluorophore) absorbs a light photon, remains in an excited state for (typically) a few nanoseconds and then emits a lower energy photon. The fluorescence intensity of a sample therefore depends upon both the efficiency of light absorption (given by ϵ , the molar extinction coefficient of the fluorophore) and the efficiency of photon emission from an excited fluorophore (given by Q , its quantum yield). Changes in sample emission intensity therefore result from a change in either ϵ and/or Q , although many investigators incorrectly assume that any intensity change results solely from a change in quantum yield without confirming this conclusion experimentally. In fact, while intensity changes with many dyes such as NBD (7-nitrobenz-2-oxa-1,3-diazole) result primarily from alterations in Q (19), intensity changes with other dyes such as fluorescein are due to changes in absorbance that are caused by alterations in pH (20), the dye's electrostatic environment (21) and/or other effects.

Why?

Fluorescence is the most sensitive spectroscopic technique available. With the best instruments, reproducible signals can be quantified from samples containing less than 1-nM concentrations of some fluorophores (hence, less than 0.3 μM of probe per cuvette containing 300 μL), concentrations much lower than those required for EPR, NMR, CD and other spectral techniques. The fluorescence signal can also be analyzed in multiple ways, including its intensity, lifetime, energy (wavelength) and rotational freedom (polarization or anisotropy), to reveal different aspects of a structure, interaction, mechanism or process

(e.g. 5,12,22). Furthermore, fluorescence is a nondestructive phenomenon, so any signal change can be monitored as a function of time to determine its kinetics.

Another advantage of fluorescence is the paucity of natural fluorophores. Background fluorescence from even complex samples is therefore typically low. In fact, measurements of samples with low fluorescence signals are more likely to be compromised by fluorescent contaminants in materials and chemicals used to prepare the samples than by natural fluorophores. (We routinely examine new chemicals for such contamination and purchase only those with minimal background signals. For example, before buying a bulk quantity of HEPES, we typically test the background fluorescence of 5–10 lots from different suppliers. These background levels vary by more than 30-fold, and the supplier of the material with the lowest background also varies from purchase to purchase.)

The background may be significant, however, when the fluorophore signal is low and light scattering is high (e.g. with mitochondria or membrane-bound ribosomes), especially with lower resolution instruments. In such cases, it is usually necessary to prepare a sample in parallel that is equivalent except for the absence of fluorophores and to subtract this blank signal from the sample signal to correct for light scattering and background, even with the best instruments that have two monochromators in the excitation light path and at least one in the emission path (22,23). While background signals are often a significant problem with native membranes, they are rarely so with liposomes prepared from purified lipids.

Another spectroscopic technique, site-directed EPR spin labeling, has provided considerable structural information about membrane proteins (24). While some information provided by EPR and fluorescence data are the same (e.g. polarity of probe environment and its rotational freedom) and hence complementary, the two techniques are distinct and have different requirements. For example, the concentration of probes required to obtain a measurable signal is much higher for EPR than for fluorescence. Also, the nitroxide spin labels often used in EPR experiments lose their signal when reduced, and hence cannot be used in samples that require reducing agents for activity (e.g. ribosomal translation of mRNA).

How?

The most common natural fluorophores are Trp and Tyr, and one can often use this intrinsic (natural) fluorescence to monitor membrane protein structure and interactions (e.g. 12,25,26). Yet, as large proteins and multicomponent protein complexes typically contain multiple tryptophans, ascertaining which Trp(s) is (are) responsible for the observed spectral signal or change may be difficult. Tyr emission is often not visible in Trp-containing proteins because much or all of the Tyr-excited state energy is

transferred by fluorescence resonance energy transfer (FRET) (see below) to nearby Trps.

One can also covalently attach an extrinsic (non-natural) fluorophore to the polypeptide, usually by incubation of a protein with a Cys- or Lys-specific fluorescent chemical reagent under native or near-native conditions. Alternatively, one can incorporate a fluorescent probe into the protein as it is being synthesized by the ribosome. In the latter case, fluorescent amino acid derivatives are incorporated at Lys (19), Cys (Alder, Jensen and Johnson, unpublished data) or amber stop codons (27) by translating mRNAs *in vitro* in the presence of a chemically modified aminoacyl-tRNA, an approach we originated over 30 years ago (28,29). One can also engineer a sequence into a polypeptide to create a fluorescent protein under the appropriate conditions (30–32).

To obtain site-specific labeling, it is often necessary to alter the protein's amino acid sequence to limit the potential chemically reactive sites or incorporation sites to one (e.g. 8,33). For example, by creating a Cys-free mutant of PFO (wild-type PFO contains only one Cys) (8), the environment of each residue in PFO could, in principle (see below), be examined simply by replacing that residue with Cys and reacting it with a Cys-specific chemical reagent to attach a fluorophore only at that site. Single-site labeling with Cys-specific reagents can also be achieved with proteins that contain disulfide bonds (5,14,34) or buried sulfhydryls (35) because reaction rates are very slow or nonexistent with such sulfur atoms.

Where should one position the fluorescent probe in the protein? In principle, any site is possible, but in actuality, some sites cannot be used. In some cases, exchanging a Cys for the wild-type residue or attaching a dye to the Cys interferes with the folding, stability and/or activity of the protein (e.g. 8,9). While such results identify functionally important sites, one cannot use them to obtain relevant spectral data. In other cases, a Cys-substituted protein is fully active, but the Cys will not react with the probe reagent under native conditions. In such cases, trying the labeling reaction under mildly denaturing conditions using urea or guanidium to partially unfold the protein often works (e.g. 8,9). In still other cases, a fluorescent-labeled protein is fully active, but the spectral properties of the probe are completely insensitive to the structural and functional states of the protein. Such probes and probe locations are typically only useful in FRET experiments (36). In short, positioning a probe at a site that does not disrupt function and that is spectroscopically useful is largely serendipitous. A crystal structure of a protein or protein domain may guide one's selection of promising sites, but even this approach fails to predict informative probe locations with 100% accuracy.

Extrinsic probes

The large number of commercially available dyes is a blessing, but there are some realities in choosing a dye

that must be recognized. First, the spectral properties of dyes are differentially sensitive to their environment. Some dyes like fluorescein are very sensitive to pH and their electrostatic environment, while others like NBD are most sensitive to the presence of water. As we have often seen, a particular change in protein conformation or environment may elicit very different spectral changes from different dyes positioned at the same location. Thus, if the emission of one dye is not altered by an expected or known structural change or interaction, then another dye with different spectral sensitivities should be tried at the same site before trying a second probe location. After all, the goal in most situations is to identify a spectral change that correlates with a change in protein structure or interaction; the type of spectral change (e.g. an increase or decrease in intensity, lifetime, polarization, emission wavelength, FRET efficiency or collisional quenching rates) is often irrelevant as long the change can be measured reproducibly.

Second, the probe may influence protein structure or function. For example, a very hydrophobic dye such as acrylodan or coumarin may alter the conformation of the polypeptide chain to which it is attached in order to bury itself in the nonpolar core of the bilayer (37). The properties of the dye become even more important when it is attached to a small protein or peptide, and the fractional contribution of the dye to polypeptide solubility and surface area is larger. Similarly, the larger a probe, the more likely it is to interfere sterically with a protein's folding, conformation and/or interactions. An example of this concern is the currently popular green fluorescent protein (GFP) and its various derivatives (YFP, CFP etc.). These approximately 25-kDa proteins are sometimes much larger than the small proteins and polypeptides to which they are attached as probes, and it is therefore impossible to ignore the probe's potential to interfere with some or all of the structural states and interactions of the protein to which it is attached. Yet, the GFP fluorophores can be generated *in vivo* and provide a mechanism for, among other things, monitoring the localization and trafficking of GFP-labeled proteins in cells, an approach that has proven extremely successful and informative during the past decade (32).

Third, some dyes are more prone than others to non-specific noncovalent association with or adsorption to proteins or membranes. Because such dyes will contribute to the observed signal, it is essential to eliminate or minimize their presence in samples if one is to correctly interpret any observed spectral data. Even though probes bound noncovalently to a specific site are sometimes used [e.g. fluorescent-labeled nucleotides bound to proteins (38) or ethidium bound to tRNA (39)], most studies employ covalently attached extrinsic dyes to ensure that every probe in the sample is in the same location (a noncovalent association involves an equilibrium between bound and free species, so unbound fluorophore will always be present). If one uses covalently attached probes, then it is necessary

to demonstrate experimentally that all of the dyes in a purified protein sample are covalently attached to the polypeptide, usually by using gel filtration or high-performance liquid chromatography under denaturing conditions to determine what fraction of the fluorophores elute with free dye instead of the polypeptide. (Note: small molecules with substantial pi electron delocalization, such as ATP and fluorescent dyes, bind to Sephadex-type resins and often elute later than the included volume). We also routinely use gel filtration with a slow flow rate to remove unreacted dyes from proteins after the modification reaction (e.g. 8) and to remove probes bound non-specifically and noncovalently to sample materials such as ribosomes (19).

Sample homogeneity and functionality

Despite the apprehension that many students and trainees have about the biophysical equations and quantitative nature of many fluorescence experiments, the reality is that the most difficult and critical aspect of every fluorescence experiment is the biochemistry, not the spectroscopy. The reason is that, except in a true single-molecule experiment, the observed signal is a combination of the individual signals from more than 10^{11} separate fluorophores. Proper interpretation of the signal and observed changes therefore depends, absolutely, on biochemical and chemical analyses of the degree of homogeneity in the sample. Does every protein in the sample contain a fluorophore? (This is not important if the presence of unlabeled proteins has no effect on the fluorescence or the process under investigation). Is each fluorophore attached covalently to the protein and at the same location? Is each protein in a sample bound to or embedded in the membrane? Is each protein in the same conformation and state of assembly? If not, then the fraction of proteins in each structural, functional and assembly state must be determined and the fluorescence signal analyzed accordingly. If this is not done and an investigator instead assumes that each protein in his/her sample is functional in every respect, labeled equivalently and in the same structural state, then the chances of misinterpreting the spectral data are substantial, especially with complex multicomponent biological systems. I cannot stress enough the importance of doing the proper biochemical and chemical controls and analyses to ensure that the spectral data are interpreted correctly.

Of course, the most important question is whether every fluorescent-labeled protein is functionally active. An experimental demonstration that each fluorescent-labeled protein functions normally, at least through some stage of a process, is the most convincing evidence that the observed spectral data are physiologically relevant. If some fraction of the labeled proteins do not function (e.g. do not bind to a membrane surface), then it is best to design the experiment to include a step or procedure that removes the nonfunctional proteins. For example, proteins or ribosome-bound nascent chains that bind stably and very tightly to a membrane surface can sometimes be separated from unbound

material by gel filtration (e.g. 19). Alternatively, one must experimentally determine what fraction of the fluorophores occupies each functional state. In short, it is the responsibility of each investigator to experimentally assess and document the homogeneity and functional status of probes in each fluorescence experiment and of each reviewer to insist that documentation of such analyses be included in a manuscript to justify its interpretations.

Which Segment(s) of a Protein is (are) Exposed to or Embedded in the Nonpolar Core of the Bilayer?

This question confronts everyone who studies a protein that functions at a membrane. Hydrophathy plots are routinely used to identify the putative transmembrane α -helices of integral membrane proteins, but it is often unclear whether the residues at the ends of these predicted TMSs are in an aqueous or nonpolar environment in the folded and assembled protein. For peripheral membrane proteins, the polypeptide sequence(s) that contacts the bilayer is rarely evident by primary sequence analysis. Thus, this information must be obtained by experiment.

Water-sensitive dyes identify aqueous versus nonaqueous environments

The most direct method to determine which residues are inserted in or exposed to the nonpolar core of the bilayer is to attach a water-sensitive fluorophore to a Cys substituted for the residue of interest (Figure 1A). An excellent extrinsic probe for this purpose is NBD: it is very small for a fluorescent dye and hence least likely to present steric problems; it is uncharged; its fluorescence properties change dramatically upon moving from an aqueous to a nonaqueous milieu; and its N and O atoms give the dye sufficient polar character to interact with and be soluble in water. 7-Nitrobenz-2-oxa-1,3-diazole's ability to exist stably in either aqueous or nonaqueous environments is critically important because it means that the NBD probe will accurately report its presence in either milieu with little bias, while probes that are either charged or very nonpolar may cause a polypeptide segment to move into an aqueous or nonaqueous, respectively, environment (e.g. 37).

The emission lifetimes and intensities of NBD, Trp and most other dyes are higher, and their maximum emission wavelengths are lower (blue-shifted), in nonaqueous than aqueous environments. Thus, one can quickly ascertain whether such a dye moves into or is exposed to the nonpolar core of the bilayer by determining whether sample emission blue-shifts and its intensity increases upon adding membranes. But, because the observed intensity or maximum emission wavelength includes signals from all of the individual probes in a sample, the distribution of probes in different environments cannot be determined unambiguously from these measurements (although it is frequently assumed that there are only two states). For

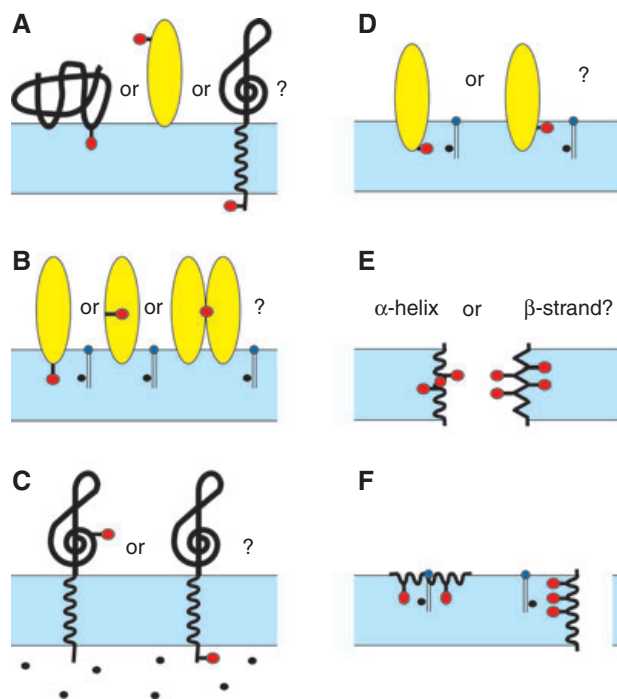


Figure 1: Protein exposure to the membrane interior. An NBD or similar dye (red) is either covalently attached post-translationally to a Cys substituted for a residue in a protein (yellow or black line) bound to a membrane (blue) or co-translationally incorporated into a protein using a chemically modified aminoacyl-tRNA. In principle, a fluorescent probe can be positioned in place of any amino acid to identify its environment at the membrane. A) The exposure of the dye to the nonpolar membrane interior or to one side of the bilayer or the other is determined by the dye's fluorescence lifetime. B) The exposure of a dye to the nonpolar membrane core is shown by a reduction in emission intensity or lifetime caused by collisions with a quencher moiety (black dots) restricted to the membrane interior by covalent attachment to a phospholipid acyl chain. Dyes buried inside a protein or between two proteins may be in nonpolar environments but will not be collisionally quenched by NO-phospholipid (PL). C) The exposure of a dye in an aqueous environment to one side of the membrane or the other is detected by collisional quenching of dye emission by a hydrophilic collisional quencher (black dots) that cannot pass through the lipid bilayer. D) The average depth of dye placement within the bilayer is estimated by quantifying the reduction of dye emission by quencher moieties located at different sites along the PL acyl chain. Maximal collisional frequency, and hence quenching, will be observed when the dye and quencher are located close to the same depth below the surface. E) The secondary structure of the protein segment that forms a pore in the membrane or binds to the membrane surface (not shown) is determined by the pattern of nonpolar exposure of a sequence of adjacent residues (although the cartoon shows multiple probe sites per polypeptide, only one site per protein is examined per experiment). The residues in a β -strand will alternate nonpolar and aqueous environments, while the same residues in an α -helix would show a helical wheel pattern of aqueous and nonpolar environments. F) A dye positioned at various nonpolar-exposed sites along a β -strand at the membrane surface would be quenched to the same extent by a quencher attached near the center of the bilayer. The same is true for an α -helix if the dyes are located at approximately the same radial position in the helical wheel. In contrast, the extent of quenching of dyes located at different depths in the membrane would differ for the same NO-PL (again, only a single probe location per polypeptide is examined per experiment).

example, a twofold increase in sample intensity may result because the emission intensity of each probe in the sample increased by twofold, or because the intensity of 50% of the probes was unchanged and that of the other 50% was increased by threefold, or any number of other possibilities. In addition, a significant intensity increase will not be observed if a probe moves from the nonpolar interior of a folded protein to the nonpolar core of the bilayer.

Thus, it is much more informative to determine the fluorescence lifetimes of the probes in a sample because a dye in a particular microenvironment will have a specific lifetime that reflects its exposure to water and other molecules. While a given lifetime does not uniquely specify a dye's environment, the observation of two different lifetimes demonstrates that the dye is present in at least two different environments. Furthermore, fluorescence lifetimes characteristic of specific environments can identify the nature of the probe's environment. For example, NBD has a lifetime of approximately 1 ns in an aqueous milieu and approximately 8 ns in a nonpolar milieu (5,19). Hence, by quantifying the distribution of NBD lifetimes in a sample, one can determine what fraction of the NBD dyes in the sample are in an aqueous or nonaqueous environment (8–10,12,18,19,23). Lifetime measurements therefore not only identify a probe's environment but also the homogeneity (or not) of the probe locations within a given sample.

Collisional quenching distinguishes the nonpolar interiors of proteins and membranes

When an excited fluorescent dye collides with some molecules and ions, its excitation energy is lost and no fluorescent photon is emitted. This phenomenon, termed collisional quenching of fluorescence, provides a direct method for determining accessibility. If the dye and quencher moiety are able to contact each other dynamically, emission intensity and lifetime will decrease. But if dye and quencher are in different compartments or locations (e.g. aqueous versus nonaqueous or cytosolic versus lumenal), the presence of the quencher in the sample will not reduce dye emission (9,18,19,21). To quantify quencher accessibility to probes, one must compare the bimolecular quenching constants (k_q), not the usually measured Stern–Volmer constants (K_{sv}) because the latter does not correct for differing excited state lifetimes in the absence of quencher (10,17,18). However, because the observed quenching is sensitive to a number of (sometimes unrecognized) effects (e.g. electrostatic effects on charged quenchers, heterogeneity in dye and quencher location, diffusion rates, static quenching, steric effects that alter collisional frequency etc.; 17), quenching data should be interpreted cautiously. In fact, it is often best to focus on a simple question: Do the dye and quencher collide or not? This simplified approach is particularly useful when the hydrophilic or lipophilic properties of the quencher restrict it to an aqueous or nonaqueous milieu, respectively, within the sample.

The movement of a probe into a nonpolar milieu could result from its burial in the nonpolar interior of a protein, its movement into a nonpolar interface between two proteins that associate or its exposure to the nonpolar core of the membrane (Figure 1B). Because the dye ends up in a nonpolar milieu in each of these possibilities, they may not be distinguished by fluorescence lifetime measurements. Thus, an independent approach must be used to determine directly which possibility is correct. A nitroxide quencher moiety (NO) is covalently attached to an acyl chain of a phospholipid (PL) to localize the NO within the nonpolar interior of the membrane bilayer. If the emission intensity or lifetime of an intrinsic or extrinsic dye on a protein is reduced when 10–20 mole% NO-PL, but not PL, is added to or included in native or liposomal membranes, then the dye must collide with the NO and be exposed to the membrane interior. But if the dye is not quenched by NO, then the dye is inaccessible from the bilayer core and presumably resides within a proteinaceous nonpolar environment. This approach has been used to demonstrate directly that specific residues within PFO are exposed to and in contact with the nonpolar interior of the membrane (8–10,12,18).

To Which Side of a Membrane is a Protein Residue Exposed?

Charged collisional quenchers such as iodide ions do not pass through the nonpolar core of a membrane at a detectable rate (23), so they provide a very direct method for ascertaining on which side of membrane a protein residue is located. When a protein with a fluorophore at an aqueous-exposed site is either inserted or co-translationally integrated into a membrane (Figure 1C), the addition of iodide ions to the sample would quench fluorophore emission only if it were exposed on the outer surface of a liposome or the cytoplasmic surface of an ER microsome or bacterial inner membrane vesicle (19,23,33,40–43). A fluorophore exposed to the aqueous interior of the liposome or microsome would not be quenched until the charged quencher moieties were introduced into the liposome or microsome interior by treating it with a pore-forming protein such as PFO, SLO or melittin (23,33,40–43).

Another approach for assessing dye exposure to the medium is to add antibodies that bind specifically and with high affinity to the dye (e.g. fluorescein, NBD or BODIPY) and quench its emission by 85–90% (e.g. 16,40). Such antibodies, some of which are available commercially, therefore provide not only a method to determine what fraction of dyes in a sample are sufficiently exposed to the solvent to bind an antibody but also a means to greatly reduce the contribution of externally exposed probes (e.g. those adsorbed to the outer vesicle or microsomal surface) to the observed sample signal.

How Deeply in the Bilayer Core is a Particular Residue Located?

Because the collisional frequency between a dye and a quencher will be greatest when they are located at the same depth within the bilayer, the approximate location of a dye, and hence the residue to which it is attached, can be estimated by determining which NO location in the PL acyl chain gives maximal quenching (Figure 1D). This approach does not provide high resolution information because of the dynamic nature of the PL acyl chains in the bilayer and amino acid side chains that tether the dye to the polypeptide backbone, but it does indicate whether the probe is located near the membrane surface or is deeply buried in the bilayer (9,10,18).

What Secondary Structure is Adopted by a Membrane-Interacting Amphipathic Sequence?

Bacterial toxins create holes in membranes by the insertion of either α -helices (e.g. 44) or β -barrels (8,9,45). To determine whether the amphipathic polypeptide conformation is α -helix or β -hairpin (Figure 1E), one can position an NBD-Cys in place of each residue of a TMS or amphipathic sequence in turn, and the pattern of NBD exposure to the membrane interior from these sites is determined by emission lifetime, intensity and NO-PL quenching. If the polypeptide segment is in a β -hairpin conformation, then an alternating pattern of aqueous and nonaqueous NBD environments would be observed (8,9,18). Similarly, probes with the same orientations in an α -helix helical wheel would be quenched equivalently. Alternatively, if the exposure of NBD to the nonpolar bilayer core correlates with that expected from a helical wheel analysis, then the polypeptide that separates the aqueous and nonaqueous phases is folded into an α -helix (18).

Does an Amphipathic Polypeptide Span the Bilayer or Lie on the Membrane Surface?

An amphipathic β -strand or α -helix may lie on the membrane surface or may span the bilayer in a transmembrane orientation if the protein forms a pore in the bilayer (Figure 1F). By replacing, one at a time, the amino acids that are exposed to the bilayer interior on one side of the helix or β -strand with the same residue (e.g. NBD-Cys) and then determining the extent of quenching of each by an NO-PL with the NO moiety positioned near the center of the bilayer (9,18), one can determine whether the β -strand or α -helix is oriented parallel or perpendicular to the plane of the membrane. If the extent of quenching is about the same for all probe sites facing the bilayer, then the β -strand is lying on the surface because each probe extends the same distance into the bilayer and is quenched to the same degree by the NO that is located at

the same average depth in the bilayer. Similarly, probes with the same orientations in an α -helix helical wheel would be quenched equily. Alternatively, if substantial differences in the extents of quenching by the same NO-PL are observed for different probe locations, then the probe sites are located at different depths within the bilayer relative to the depth of the NO quencher. In this case, the β -strand or α -helix must span the membrane (9,18).

Which Domain of a Peripheral Membrane Protein Contacts the Bilayer First?

Because fluorescence signals can be monitored as a function of time, the kinetics of specific spectral changes can be monitored to reveal the order of events during a process. In the case of PFO, two domains termed D3 and D4 were shown to insert into the bilayer (8,9,12), and it was therefore of interest to determine which domain interacted first with the bilayer because that domain was most likely responsible for recognizing the cholesterol in the bilayer and initiating the membrane-binding process. Because the D4 Trp emission intensity always increased before the D3 NBD intensity did, it was clear that D4 is responsible for initial membrane binding (12). As long as one can synchronize the system at some initial time, the rate of change of any spectral parameter can, in principle, be determined. Although monitoring intensity changes are the easiest, time-dependent changes in lifetime, polarization, FRET efficiency and quenching can also be quantified.

What is the Sequence of Events in a Process?

The kinetics of fluorescence changes can also be measured to establish the relative timing of structural and topographical changes and thereby reveal the mechanism(s) by which a system moves from one state to another. For example, PFO does not form oligomers until it binds to a membrane surface, suggesting the existence of a membrane-binding dependent conformational change in the protein that exposes an interfacial surface used in oligomer formation (5). When PFO was examined, an NBD-detected conformational change in D3 more than 70 Å above the surface was indeed shown to occur only upon membrane binding, and interestingly, the rate of this conformational change was indistinguishable from the fluorescence-detected rate of D4 Trp binding to the membrane (5). The conformations of D3 and D4 in PFO are therefore tightly coupled, as was also indicated by previous kinetic data (12).

What is the Spatial Separation Between Two Residues in the Same or Different Proteins?

FRET

Excitation energy is sometimes transferred from one dye to another by resonance energy transfer. After excitation

by the absorption of a photon, the donor or D dye can transfer its excited state energy to a second chromophore or dye (the acceptor or A) nonradiatively (i.e. without photon emission). The efficiency of this transfer depends on, among other things, the extent of overlap of the D emission and A absorption spectra, the relative orientation of the transition dipoles of D and A and – most importantly – the distance between D and A. FRET can measure distances between 20 and 100 Å (46), as well as detect conformational changes and determine their magnitudes (e.g. 22,47,48). Because the relative orientation of D and A cannot be determined experimentally for nonrigid systems, distances measured by FRET have some degree of uncertainty that is estimated from the measured polarization of D and A (e.g. 22,47,48). Yet, the agreement between distances determined by FRET and by crystallography are usually within 10% (46,49) [also compare (47) with (50) and (51) with (52)]. The effect of orientational uncertainty can be minimized by comparing FRET efficiencies in two samples where the same D and A have similar anisotropies. The focus is then on changes in FRET efficiency that reveal functionally important changes in structures or relationships (e.g. does a cofactor alter the height and/or orientation of an enzyme's active site above the membrane? does A bind to B?) rather than on determining a precise distance between D and A.

FRET is best detected by the reduction in D emission intensity or lifetime. One can also detect FRET by a D-dependent increase in A-emission intensity, but quantifying this increase is more problematic because of the spectral overlap of D and A emissions. While D and A are usually different, FRET also occurs between the same dyes if their emission and absorbance spectra overlap significantly [e.g. fluorescein (53) and BODIPY (54)]. Because D and A are the same, this type of FRET (termed homoFRET) is detected only by a reduction in dye anisotropy, not by reduced donor intensity or lifetime.

Operationally, accurate analytical FRET experiments require that one prepare four samples in parallel that are identical except for their dye content: both D and A, only D, only A or neither dye [i.e. unlabeled molecules replace the labeled ones (22,47,48)]. The dye-free blank signal is subtracted from those of the other three to eliminate light scattering and background signal, and the A-only signal is subtracted from that of the D + A sample to correct for direct excitation of the A dye. [Sometimes the A-only and blank signals are too small to significantly alter the D-only and D + A signals, but this must be evaluated experimentally each time, especially when samples contain membranes, ribosomes or other particles that scatter light efficiently. Blank subtraction is also often necessary for lifetime measurements in complex samples (23,55).] The net and, if necessary, normalized D-only and D + A signals (intensities or lifetimes) are then compared directly to determine the efficiency of energy transfer.

However, calculating the FRET efficiency directly from the measured net steady-state intensities of the D-only and

D + A samples can only be done if each D is paired with an A. If this does not occur (e.g. if some fraction of the D-labeled molecules are not bound to A-labeled molecules or to a membrane containing the A-labeled molecule), then one must use biochemical methods to quantify what fraction of the D dyes are actually participating in FRET and make the appropriate corrections. Frankly, it is usually much easier to design the FRET experiment initially to ensure that every D has an A partner in the sample than it is to determine biochemically what fraction do not (for more detailed comments, see 22,47). For example, if one wants to determine the distance between a protein site and a nucleotide-binding site by FRET, it is best to attach the A dye to the nucleotide (38) because excess nucleotide must be added to the sample to ensure that the nucleotide-binding site of every protein is occupied. One can then determine the FRET efficiency from the decrease in protein-bound D emission without regard for the excess unbound A-labeled nucleotides. In any event, as noted above, the accuracy of any FRET measurement of the spatial separation between a D and an A on the same (Figure 2A) or different (Figure 2B) molecules depends absolutely on knowing the biochemical homogeneity of the sample.

FRET experiments are now being done *in vivo* using derivatives of GFP covalently attached to the proteins or domains of interest, and this approach has already demonstrated its value (reviewed in 32), even though the development of analytical procedures and instrumentation is on-going (56,57). An array of biochemical sensors has been created in which D- and A-labeled proteins are linked by a peptide that undergoes a conformational alteration in response to changing cell physiology (32,56). FRET can also be used to determine the proximity of membrane proteins in living cells. Furthermore, the extent of FRET (and its reversibility) can be monitored as a function of time, thereby revealing the dynamics of D and A proximity. Yet, FRET experiments done in cells must be interpreted cautiously when D and A are in two different proteins because the concentrations of the two at any location in the cell is not fixed. Hence, unlike the covalently linked sensor proteins described above, determining the extent of FRET between separate D- and A-labeled proteins from the magnitudes of D- and A-emission intensities may not be the optimal approach. Instead, FRET is best detected and quantified by monitoring donor fluorescence lifetimes (fluorescence lifetime imaging or FLIM) (32,56,57).

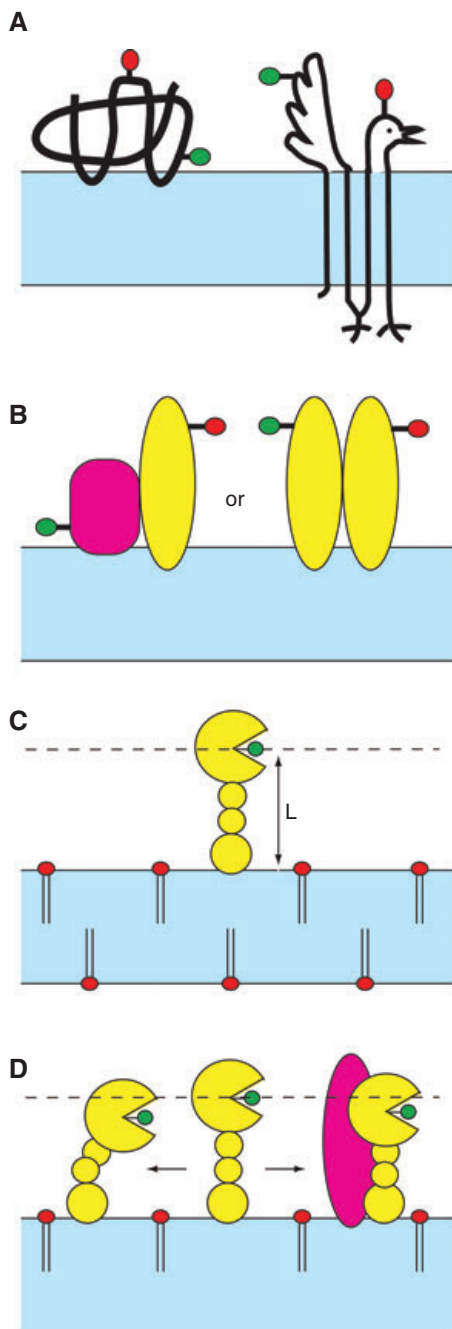
While *in vivo* FRET is usually assumed to result from the direct association of the D- and A-labeled proteins, it is difficult to rule out other possibilities. For example, D- and A-labeled proteins may be actively sequestered within the organized cellular milieu at concentrations high enough to detect FRET even when they are not associated. Alternatively, the close approach of the D- and A-labeled proteins may be mediated by their simultaneous binding

to a third component, not to each other. In short, the inability to directly evaluate biochemical homogeneity *in vivo* complicates the biochemical interpretation of spectral data.

Where is a Residue Located Relative to the Membrane Surface?

Point-to-surface FRET

A variation of the FRET technique allows one to measure the distance between two parallel planes, one of which is the membrane surface. Charged A dyes can be localized



at the aqueous–membrane interface by attachment to a PL or similar molecule (e.g. rhodamine attached to the headgroup of phosphatidylethanolamine to form Rh-PE). If all membrane-bound proteins adopt the same conformation, D dyes covalently attached to the same single site on each protein will be located at the same height above the membrane, thereby creating a second plane. When the Rh-PE molecules diffuse freely in the membrane, the distance of closest approach between the two planes (i.e. the height of the D dye above the membrane surface; Figure 2C) can be quantified using analytical expressions that integrate FRET from D dyes in one plane to A dyes that are distributed randomly and uniformly on the membrane surface (e.g. 58). Estimations of D-to-surface heights greater than about 50 Å appear to be fairly accurate (51,52,59,60) using one such expression (61), but calculations of shorter heights are complicated by several effects that become more important as D moves closer to the surface.

Topography and conformational changes determined by FRET

This approach has been used to determine the height above the membrane of a number of protein domains. For example, the active site locations of several membrane-bound blood coagulation enzymes were determined, as well as changes in active site location and/or orientation elicited by the binding of the protein cofactor required for enzyme activity (Figure 2D center, right) (36,48,51,59,62–66). The locations of different PFO domains above the membrane surface at different stages of pore formation have also been determined, thereby demonstrating that the elongated PFO molecule is initially oriented perpendicularly on the membrane, with only the tip of D4 contacting the surface (Figure 2C) (10,67). This study also revealed that PFO undergoes major conformational changes upon insertion into the membrane, with some segments of the protein moving more than 60 Å and other domains moving about 40 Å (Figure 2D left, center) (67).

Figure 2: Protein structure determined by fluorescence resonance energy transfer (FRET).

A) D (green) and A (red) are incorporated into specific sites in a single protein, and the FRET efficiency indicates the spatial separation of the two dyes in the protein. B) The FRET efficiency between D and A attached to two different proteins (yellow and magenta) or in two different derivatives of the same protein (yellow) indicates the separation of the dyes in a heteromultimeric or homomultimeric protein complex. C) The height of D above the membrane surface is determined by a variation of the FRET technique that localizes the A dyes at the surface and measures the point-to-surface separation. The dashed line represents the plane formed by the D dyes, and L is the distance of closest approach between the two planes. If L is large, the transfer of energy from D to A dyes on the other side of the bilayer is very small and can be neglected. D) Changes in topography can be elicited either by conformational changes that occur as a protein moves between stages of a multistep process (center to left) or by association with another protein (magenta) that causes D and the domain to which it is attached to change its height and/or orientation above the membrane surface (center to right). Other molecular species are as defined in Figure 1.

This approach can therefore provide structural, topographical and mechanistic information about the interaction of a protein or protein complex with the membrane. Interpretations are simplest when examining proteins bound to liposomes in which Rh-PE distributes randomly and equally on the membrane surface, as in the above cases. For experiments using native membranes, where membrane proteins occlude a variable amount of surface area, the surface density of A must be determined experimentally (68,69).

What is the Quaternary Structure of Membrane-Bound Complexes?

The arrangement of proteins and domains (and their stoichiometry) in multicomponent complexes, as well as the magnitude of significant conformational changes, can be determined relative to each other and to the membrane using the point-to-point and point-to-surface FRET techniques described above if one is able to reconstitute functional complexes with one or two fluorescent-labeled proteins. For example, FRET between fluorescent-labeled SecY derivatives showed that in a membrane, SecYE associates to form multimers containing two or more SecY molecules (70).

Are Two Residues Adjacent?

The hypothesized close approach of two specific sites in protein complexes (or in a protein upon folding) can be tested directly by replacing the residue at each site with a Cys and labeling the Cys with pyrene. If the residues are juxtaposed upon the association (or folding) of the proteins, then the aromatic pyrenes may stack and form an excimer with an altered emission spectrum (5). While excimer formation conclusively demonstrates the close approach of the two sites in the protein or complex, the absence of excimer formation does not prove that the sites are significantly separated in the complex because the pyrene dyes are large and may have restricted rotational freedom around their tether to the protein, thereby preventing their stacking even when adjacent.

Monitoring Co-translational Membrane Protein Biogenesis, Folding and Assembly

The movement of a fluorophore into a different environment often elicits a spectral change that can be correlated with the movement of the dye-bound molecule into a different structural or functional state. Fluorescence therefore allows one to characterize changes in protein conformation and/or environment during biogenesis, including the kinetics of specific steps in a process. For example, the folding of a single polypeptide, the association of that polypeptide with another protein to form a complex and/or

protein exposure to or insertion into the bilayer core can be detected by changes in fluorophore emission intensity (Figure 3A). As another example, the timing of nascent chain loop exposure to the cytosol during co-translational integration at the ER membrane can be determined as a function of nascent chain length by collisional quenching (Figure 3B) (33). Such changes can be used to characterize the biogenesis, folding and assembly of membrane proteins, but identifying a spectroscopically useful signal change is generally serendipitous, particularly in samples containing multiple proteins and native membranes. It is therefore best to examine all fluorescence properties of a probe, including its intensity, polarization, lifetime and accessibility, at each stage of the process being examined before choosing the property on which to focus.

Sometimes, the most direct approach to monitor membrane protein folding and assembly, or the lack thereof, is to use FRET to detect the close approach of two sites in the same polypeptide as it folds (Figure 3C, left) or of two sites in different proteins as they associate to form a complex (Figure 3C right). The FRET-dependent decrease in D emission can be monitored both as a function of time to determine the kinetics of the process and also as a function of the maximal D emission change to determine the extent of completion if the process has only two states (e.g. associated and nonassociated; note that a large protein may pass through several intermediate states during folding, and if the FRET efficiencies differ for those states, the extent of completion will be difficult to determine).

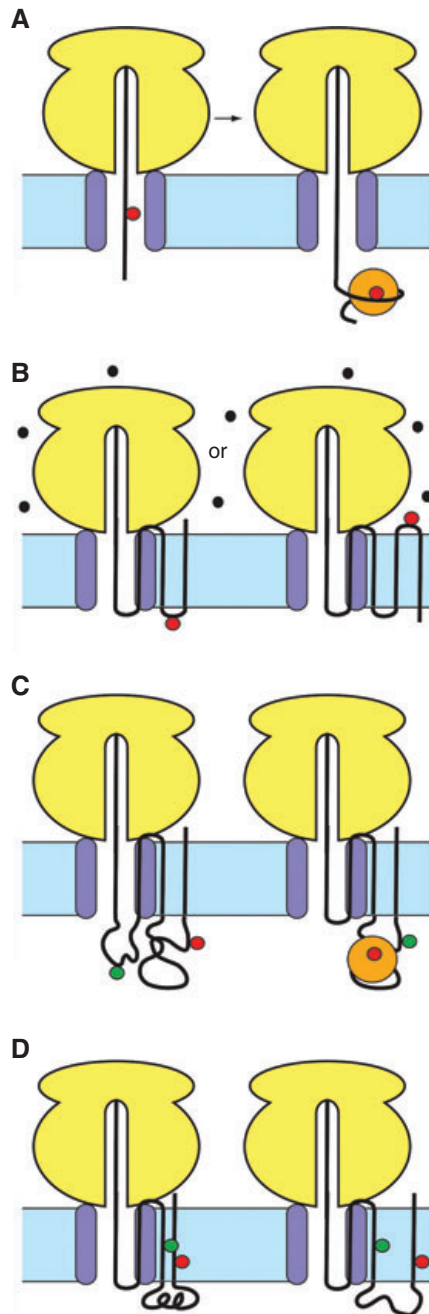
The folding of nascent membrane proteins inside the ribosome has been detected by FRET between a D and an A incorporated into the same nascent chain using fluorescent-labeled aminoacyl-tRNAs (cf. Figure 3C, left). Specifically, the folding of the nonpolar TMS of a nascent membrane protein into an α -helix (or nearly so) was induced by the ribosome (22). In contrast, FRET measurements showed that two secretory proteins were fully extended, or nearly so, inside the ribosome (22). This study therefore not only demonstrated the efficacy of this approach but also its experimental potential (see 71). For example, this approach can be used to monitor the co-translational proximity of a D and an A at two sites of interest in a nascent protein at different stages of its integration and assembly [e.g. two different TMSs within or close to the translocon (Figure 3D), or two loops thought to associate during folding (Figure 3C, left), or nascent chain association with a chaperone or processing protein (Figure 3A, right) (72)].

How to Detecting and Quantifying the Binding of Proteins to Other Molecules?

When the binding of a protein to another protein, membrane, nucleic acid or small molecule causes a

fluorescence change, the alteration in emission intensity (Figure 4A), anisotropy (Figure 4A) (especially when small fluorescent-labeled ligands bind to large proteins; 38) or FRET efficiency (Figure 4B) can be used to characterize that association as a function of time, component concentration or another variable, thereby allowing the quantification of the kinetics and thermodynamics of the association (27,73–76).

To determine most accurately the affinity of two molecules, it is necessary to measure the K_d at equilibrium.



Non-equilibrium techniques estimate the extent of binding in a sample by first separating the bound complex from the unbound species and then measuring the amount of complex. Yet, because the complex dissociates during the separation procedures, K_d values calculated from such data are typically much higher than the true K_d values. For example, the K_d values for aminoacyl-tRNA·EF-Tu·GTP ternary complexes determined using nonequilibrium techniques were 10- to 1000-fold higher than the actual equilibrium K_d (73,74).

The optimal approach for quantifying the amounts of bound and unbound species in a sample at equilibrium is to use a nondestructive spectroscopic technique that can monitor and distinguish between the bound and free species in a cuvette without separating them. The fraction of bound fluorescent-labeled ligand in a sample is then given by the observed fraction of the maximal spectral change. By titrating the unlabeled species into a sample containing the fluorescent-labeled species, one can easily determine the dependence of complex formation on ligand concentration. K_d values are determined experimentally from this concentration dependence. Accurate measurements require that samples contain measurable amounts of both free and bound ligands. This requirement greatly complicates the K_d determination of high affinity interactions ($K_d < 10$ nM) because significant amounts of both free and bound species would be observed only in samples containing nM concentrations of the ligands. For this reason, fluorescence is the only acceptable choice for such a measurement because it is the only spectroscopic technique that can accurately detect and monitor probe concentrations that are nM or lower (e.g. 27).

Finally, it is important to emphasize that one can spectroscopically determine the true K_d value for a receptor (R) binding to a natural, unmodified and nonfluorescent ligand

Figure 3: The co-translational environments of nascent proteins at a membrane. A) Some spectral properties of a dye incorporated co-translationally into a nascent protein may change as the dye moves to a different environment or compartment (left) or interacts (right) with other proteins (orange). The translocon, the site of protein translocation through or integration into the membrane (77), is represented by the purple ovals and its aqueous pore by the white separator. B) The exposure of a dye in a nascent protein to one side of the membrane or the other (or to neither) can be determined by collisional quenching with hydrophilic quenchers that do not cross the bilayer. The black dots represent collisional quenchers such as iodide ions. C) The spatial separation between a D (green) and an A (red) dye determined by fluorescence resonance energy transfer (FRET) indicates the extent of nascent chain folding at different stages of biogenesis (i.e. nascent chain lengths) (left) or of nascent chain association with a protein (orange) labeled with an A dye (right). D) The separation between different pairs of transmembrane segments at different stages of membrane protein biogenesis and assembly can be monitored by FRET. Other molecular species are as defined in Figure 1.

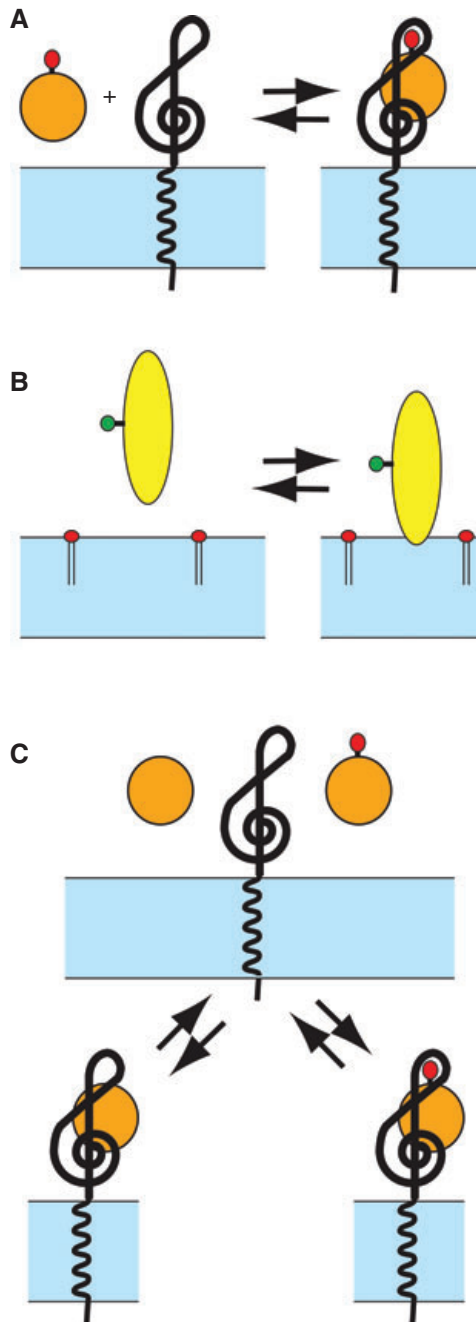


Figure 4: Fluorescence-detected and fluorescence-quantified association of molecules. A) When the association of a protein with a membrane, small ligand, RNA, DNA or another protein (orange) results in a measurable change in fluorophore (red) intensity or anisotropy, the dependence of that association can be monitored as a function of concentration, time or other variable. B) The association of a protein with another molecule(s) (here a membrane) can be detected and quantified by changes in FRET efficiency. C) To determine the true K_d value for a natural, non-fluorophore-labeled species, one can determine its ability to compete at equilibrium with the fluorescently labeled species for binding to the common receptor. Other molecular species are as defined in Figure 1.

(L) by the ability of L to compete with a fluorescently labeled L analog (FI-L) for binding to R. When R is added to a sample (a cuvette) containing both L and FI-L, two competing binding equilibria are established that reflect the relative affinities of R for L and FI-L (Figure 4C). Because the amount of R·FI-L complex in a sample is given directly by the magnitude of the observed spectral change, the extent of competition by nonfluorescent L for binding to R is given by the extent to which L reduces fluorescence-detected R·FI-L complex formation. The equations representing the two equilibria for competitive R binding to L and FI-L can be solved simultaneously from the extent of spectral change, the known total concentrations and the spectroscopically determined K_d for R·FI-L, because the free R concentration is the same for each equation at equilibrium. Because the total R and free R concentrations are not the same in a high-affinity interaction, an exact equation that relates the observed fluorescence change to the known total concentrations of the components and the binding parameters must be used (e.g. 27). Using this approach, K_d values for protein binding to natural, unmodified ligands can be quantified directly; the fluorescently labeled molecules serve solely to quantify the distribution of receptor within the sample, and their presence does not alter the measured K_d provided ligand binding is nonco-operative and independent.

Do Proteins Form a Pore in the Membrane, and if so, What is its Size?

Many proteins create holes in membranes (e.g. 3,77), and fluorescence can be used to characterize both the proteins (see above) and the pore. The most direct way to detect pore formation is to encapsulate a fluorophore or fluorescently labeled molecule in a liposome or microsome (16,40,43). After purification by gel filtration to remove nonencapsulated fluorophores, a quencher is added to the sample, followed by the protein being investigated (Figure 5A). If the protein forms pores large enough to release the fluorophore-labeled species or allow entry of quenchers into the vesicle interior, then fluorophore exposure to the quencher causes a reduction in intensity that can be monitored as a function of time (16). Thus, not only does this approach reveal whether a pore is made but also the kinetics of pore formation.

The size of the pore can be estimated by encapsulating fluorescently labeled molecules of different sizes (16,40). For small holes, one can encapsulate $[\text{Tb}(\text{dipicolinate})_3]^{3-}$ and use EDTA as the quencher (Figure 5A). Alternatively, one can use fluorescein- or BODIPY-labeled species of different sizes and add dye-specific antibodies as the quencher to determine the approximate size of molecule that will pass through the pore and whether the different-sized species are released at the same rate (Figure 5B) (16). One can also estimate translocon pore size by using collisional quenchers of different sizes and determining

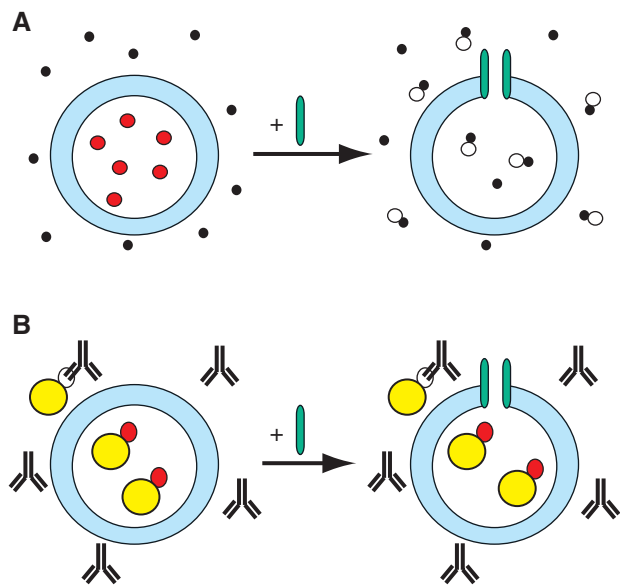


Figure 5: Fluorescence detection and sizing of pores. Liposomes or microsomes encapsulating fluorophores of different sizes, from A) [Tb(dipicolinate)₃]³⁻ (red) to B) fluorescein- or BODIPY-labeled (red circles) molecules of different sizes (yellow), are added to solutions containing quenchers of the fluorophores [EDTA (black dots) in A or antibodies to fluorescein or BODIPY in B]. If a protein (green) added to the solution creates a pore in (A) large enough to pass EDTA or [Tb(dipicolinate)₃]³⁻, EDTA will replace dipicolinate as the chelator of Tb³⁺, thereby greatly reducing its emission intensity (○). Note: Tb³⁺ binds tightly to some proteins and may fluoresce due to FRET from Trp (25,26), thereby complicating data analysis. If the pore created is too small to allow the quencher or fluorophore to contact each other (B), then no quenching is observed upon pore formation. By varying the size of the molecule to which the dye is attached (yellow; B), one can estimate the diameter of the pore. After vesicle purification, any residual nonencapsulated and exposed fluorophore (○, in B) will be quenched by an antibody before the putative pore-forming protein is added. Other molecular species are as defined in Figure 1.

which are able to move through the translocon to reach a nascent chain fluorophore inside the ribosome on the other side of the membrane (40).

Conclusion

This brief summary has provided an indication of the wide range of fundamental questions about membrane proteins that can be addressed, in most cases uniquely, using fluorescence spectroscopy. While the decision to embark on fluorescence-based studies of a membrane protein(s) may be difficult because of the investment in instrumentation, time and training that is necessary, the potential rewards are enormous. The truth of this conclusion is amply demonstrated by the dramatic increase in the understanding of CDC structure, function and mechanism during the past 7 years, as well as unprecedented insights

into various aspects of protein trafficking at membranes that have been provided by fluorescence experiments. The desire to understand various aspects of a membrane protein at the molecular level may therefore make such an investment eminently worthwhile and valuable.

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