Review
Fluorophores, Environments, and Quantification Techniques in the Analysis of Transmembrane Helix Interaction Using FRET

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ABSTRACT:

Förster resonance energy transfer (FRET) has been widely used as a spectroscopic tool in vitro to study the interactions between transmembrane (TM) helices in detergent and lipid environments. This technique has been instrumental to many studies that have greatly contributed to quantitative understanding of the physical principles that govern helix–helix interactions in the membrane. These studies have also improved our understanding of the biological role of oligomerization in membrane proteins. In this review, we focus on the combinations of fluorophores used, the membrane mimetic environments, and measurement techniques that have been applied to study model systems as well as biological oligomeric complexes in vitro. We highlight the different formalisms used to calculate FRET efficiency and the challenges associated with accurate quantification. The goal is to provide the reader with a comparative summary of the relevant literature for planning and designing FRET experiments aimed at measuring TM helix–helix associations. © 2015 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 104: 247–264, 2015.

Keywords: FRET; transmembrane; oligomeric state; helix; lipids and detergents

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INTRODUCTION

Integral membrane proteins are extremely important biologically. They comprise a quarter to one-third of all proteomes[1] and perform multiple important biological functions such as signaling, material transport, and defense. They are also the target for more than half of the currently marketed drugs.[2]

Structurally, there are two main classes of integral membrane proteins, the helical and the barrel proteins.[3] The more common helical class spans the lipid bilayer with transmembrane (TM) helices that typically comprise 20–30 hydrophobic amino acids. The helical membrane proteins are either designated as bitopic, which have a single TM helix, or as polytopic, which have multiple TM helices folded into a bundle. The TM beta barrel occurs in the outer membrane of bacteria and organelles of endosymbiotic origin, mitochondria, and chloroplasts. Beta barrels often form channels and pores, with hydrophobic residues pointing toward the lipid region and more polar residues toward the hydrated pore of the barrel.

Structural information serves as a nucleation point to understand the functional mechanism of any macromolecular system, but because membrane proteins are embedded in a chemically heterogeneous lipid bilayer, their biophysical characterization can be very challenging. The structural investigation of membrane proteins is becoming more feasible because of the developments in the overexpression of proteins, their solublization, and ways of increasing protein stability.[4,5]

Nevertheless, the rate at which the structure of membrane proteins is solved still lags behind that of soluble proteins.[6]

Along with structural studies, research has focused on understanding oligomerization, which is increasingly seen as a common occurrence in the case of membrane proteins.[7] An important foundation for these studies has been laid two decades ago by the Two-Stage Model,[8] which frames the membrane protein folding problem into two thermodynamically independent stages: (1) the insertion of individual TM helices into the bilayer to form stable independent helices; and (2) the association between these individual helices to form the final folded tertiary structure or to form quaternary oligomeric complexes.

This model suggests that in the case of membrane proteins, both the folding and the oligomerization processes share a common central event, that is, the lateral association of individual TM domains. Thus, study of the oligomerization of these helices is a way to understand the folding principles of membrane proteins. Even though the Two-Stage Model may be oversimplified and not take into account the complexity of biological insertion and potential transitional states during insertion or postinsertion, much of the existing structural and functional data provide general support for its basic thermodynamic assumptions.[9] Based on these data, the study of TM helix oligomerization has contributed to significant progress in understanding the principles and motifs involved in the folding of the membrane.[10]

METHODS FOR STUDYING HELIX–HELIX ASSOCIATIONS: BIOPHYSICAL METHODS *IN VITRO*

A number of biophysical methods have significantly contributed to understanding the energetics of TM helix oligomerization, such as Förster resonance energy transfer (FRET),[11–15] sedimentation equilibrium analytical ultracentrifugation (SE-AUC),[16,17] SDS-PAGE,[18,19] disulfide exchange equilibrium,[20,21] and steric trapping.[22,23] Among these methods, the two most widely used are SE-AUC and FRET. SE-AUC is directly sensitive to the oligomeric mass of an associated complex and is a rigorous technique that can directly measure the stoichiometry as well as the equilibrium constants of an oligomer.[16] Unlike FRET, SE-AUC does not require the introduction of labels, which could potentially interfere. However, one important limitation of SE-AUC is that measurements can only be performed in detergent micelles. Therefore, FRET has been particularly important for investigating the energetics of association of TM helices in lipid bilayers, which are a better approximation to the natural membranes in which these helices reside.

FRET: A POWERFUL AND VERSATILE METHOD FOR STUDYING MEMBRANE PROTEIN INTERACTION *IN VITRO* AND *IN VIVO*

FRET uses proximity as a means to detect TM domain interactions and is compatible with both detergent and lipid milieus. As mentioned, the incorporation of an artificial label can potentially alter the natural course of an interaction; however, the effect of the labels can be accounted for with proper controls. FRET is an exceptionally versatile technique for studying the interactions between synthetic peptides labeled with organic dyes in artificial bilayer mimetics, as well for investigating proteins in live cells using fluorescent protein fusion constructs. Ranging from qualitative[24] and quantitative[25–28] ensemble average spectroscopic data, to kinetic time-lapse FRET measurements,[29,30] all the way to FRET under the microscope[31–33] with sophisticated spatial and temporal resolution data, an extensive body of studies have been developed to study a variety of different interactions within a membrane[34]: interactions between lipids, between lipids and proteins, and between proteins.

In this article, we review the use of steady-state FRET in the literature to investigate interactions in membrane proteins, with a particular focus on the interaction of the single TM helices of bitopic membrane proteins. These oligomeric complexes have been a favorite system for investigating the rules that govern TM helix association.[10,35–38] In addition to being a tractable system, the bitopic membrane proteins attract interest because of their biological importance. These proteins comprise the most numerous class of membrane proteins (constituting about half of the total[1,39,40], and their oligomerization plays important roles in assembly, signal transduction, ion conduction, and regulation in a wide variety of biological processes as has been extensively reviewed.[38,40–43]

The discussion will begin with a brief introduction to fluorescence and FRET. We will then introduce the use of this technique in studying integral membrane proteins and systematically cover the different model membranes and detergents used in these studies. This will include a discussion on the important contributions to this field to investigate specific proteins or protein families or to improve on existing FRET methodologies and formalisms, or both. In subsequent sections, we will enlist the common formalisms used to calculate FRET efficiency and factors that can alter the observed FRET efficiency dramatically, including choices of fluorophores used. Finally, we will discuss the utility of FRET to quantify the oligomeric state of integral membrane proteins, a task that is still challenging. The goal of this review is to provide the reader with a comparative summary of the relevant literature for planning and designing FRET experiments for determining

TM helix–helix interactions. Table I provides a schematic overview of this literature, listing the membrane mimetics, the fluorophores, the FRET methods, and the biological systems that are the subject of these studies.

FRET: A TOOL TO INVESTIGATE MEMBRANE PROTEIN INTERACTIONS

FRET to Study Macromolecular Association

FRET is a photophysical process that is related to the phenomenon of fluorescence. FRET occurs when an electronically excited donor (*D*) fluorophore is quenched by a nonradiative energy transfer to an acceptor (*A*) chromophore in the vicinity, instead of emitting fluorescence (Figure 1a). The acceptor chromophore does not need to be fluorescent but often it is fluorescent, in which case, FRET can be detected by increased fluorescence at the emission wavelengths of the acceptor. FRET is possible if the *D–A* pair has a dipolar overlap (the emission spectrum of *D* overlaps with the excitation spectrum of *A*; Figure 1b) and if the two fluorophores are within 10–100 Å of each other. This range depends on the distance parameter specific to each *D–A* FRET pair, called the Förster radius (R_0). The Förster radius is defined as the distance between *D* and *A* at which the FRET efficiency *E* is 50%:

$$E = \left[\frac{R_0}{(R_0 + r)} \right]^6, \tag{1}$$

where *r* is the distance between the pair. Thus, FRET measurements could in principle yield distance measurements between two fluorophores, although this is very difficult in practice. R_0 can be calculated as follows:

$$R_0 = \left[(Q_0 J n^{-4} K^2)^{1/6} \times 9.7 \times 10^3 \right], \tag{2}$$

where Q_0 is the quantum yield of the donor in the absence of the acceptor, *J* is the spectral overlap integral, *n* is the refractive index of the medium, and K^2 is the dipole–dipole orientation factor.[25] The calculated R_0 values, assuming random fluorophore orientation, typically lie within the 15–60 Å range, which is comparable with the size of biological macromolecules (30–50 Å), and thus, FRET has been widely used as an indicator of proximity to elucidate macromolecular association and major conformational changes.

FRET Studies of TM Interaction: Progress Over the Years

Characterization of membrane protein interactions using FRET between labeled proteins or peptides has already been in use for over three decades. The earlier studies show examples of both qualitative and quantitative measurements. In 1977, a quantitative approach by Veatch and Stryer looked at the dimerization of gramicidin A25 and derived formalisms for distinguishing different oligomeric schemes. In 1982, Morris et al.[24] qualitatively used FRET to show that chromaffin granule membrane proteins form aggregates on addition of calcium. These early studies paved the platform for use and improvement of FRET techniques to study membrane proteins. In 1994, Adair and Engelman[26] derived a model for the energy transfer within oligomers and used it to show that the glycophorin A (GpA) TM domain formed a dimer, as opposed to a higher-order oligomer. This formalism has since then been widely used in quantitative FRET studies to confirm dimerization of TM helices. Further progress in assessing the oligomeric state was made by others, including formalisms applicable to higher oligomers[28,63] (see the "Determination of the Oligomeric State Using FRET" section).

These earlier studies enabled quantification of the energetics of oligomerization, but did not account for the FRET contribution due to random proximity. Simulated by Wolber and Hudson in 1979[65] and later by Wimley and White in 2000,[66] the proximity FRET term was for the first time included in membrane protein FRET efficiency calculation by Hristova and coworkers in 2005,[13] and then further improved on and extended to larger oligomers in 2014.[67] A different formalism was also developed by Fernandes et al. in 2008.[68]

These different formalisms have been applied over the years to investigate many systems and different problems involving interacting TM helices. For example, Engelman and coworkers[11,26,44] and Schneider and coworkers[45,54,69,70] have used FRET to study the dimerization of the model system GpA and its sequence variants in different membrane mimetic environments. Hristova and coworkers have contributed significantly to the field of FRET in membrane proteins by developing new experimental methodologies,[57,71,72] testing different bilayer environments,[13,60,73–76] and improving on existing formalisms for FRET calculations[13,67,77] to investigate the dimerization of proteins of the receptor tyrosine kinase family.[12,42,55,56,58,61,62,78] Deber and coworkers have studied oligomerization of natural and model peptides to understand the structure–function relationship of receptors,[50] ion pumps,[51] and other biological systems and to investigate the rules that govern membrane protein folding.[53] These and many other investigations over the years have proved FRET to be one of the most important tools to characterize the interaction of membrane proteins and yielded invaluable understanding of folding, association, and structure.

Table I FRET Studies of Transmembrane Helix Association

Donor	Acceptor	Hydrophobic Solvent	Förster Radius	Biological System	FRET Method	Reference
AMCA	DABS-chloride	SDS micelles C12E8 micelles OG micelles DOPC vesicles	49 ± 1 Å 48 ± 1 Å	Phospolamban	Donor quenching	28
DANS-chloride	DABS-chloride	SDS micelles C12E8 micelles OG micelles DOPC vesicles	33 ± 1 Å 32 ± 1 Å	Phospolamban	Donor quenching	28
Pyrene	7-Dimethylaminocoumarin	SDS micelles DDMAB micelles DPC micelles	60 Å	Glycophorin A	Acceptor sensitization	11
Pyrene	7-Dimethylaminocoumarin	C_{12} sulfate C_{12} DMAB C_{10}, C_{11} Maltoside C_{10}, C_{11}, C_{12} DAO	Not used	Glycophorin A	Acceptor sensitization	44
Fluorescein	TAMRA	DDM + SDS mixed micelles	54 Å	Glycophorin A	Donor quenching	45
Tyrosine57	Tryptophan10 Tryptophan12	DOPC vesicles SDS micelles	13.2 Å 9 Å	Bacteriorhodopsin		46
2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl] trimethylammonium (NBD)	Rhodamine	SDS micelles DMPC vesicles	50 Å	EphA1 receptor	Donor quenching	47
Fluorescein isothiocyanate (FITC)	Rhodamine	SDS micelles	55 Å	IRE1α	Donor quenching	48
DANS-chloride	DABS-chloride	SDS micelles	Not used	Major coat protein	Donor quenching	49
DANS-chloride	DABS-chloride	SDS micelles	Not used	ANTRX1	Donor quenching	50
Fluorescein amidite (FAM)	TAMRA	SDS micelles PFO micelles	Not used	Na,K–ATPase	Donor quenching	51
7-Hydroxycoumarin	FAM/FITC	DPC micelles POPC vesicles	Not used	FtsB and FtsL	Donor quenching	14
7-Hydroxycoumarin	FITC	C_{14} Betaine micelles	Not used	CHAMP peptides	Donor quenching	52
DANS-chloride	DABS-chloride	SDS micelles	Not used	Model Ai_{pep}	Donor quenching	53
DANS-chloride	4-(diethylamino) phenylazobenzene-4-sulfonyl chloride (DPBS)-chloride	DHPC vesicles	39 Å	Gramicidin A	Donor quenching	25
DANS-chloride	DABS-chloride	DMPC vesicles	40 Å	Glycophorin A	Donor quenching	26
Fluorescein	TAMRA		49–54 Å	Glycophorin A	Donor quenching	54

Table I. *Continued*

Donor	Acceptor	Hydrophobic Solvent	Förster Radius	Biological System	FRET Method	Reference
Fluorescein	Rhodamine	DMPC, diC(14:1)PC, diC(16:1)PC, diC(18:1)PC, diC(20:1)PC, diC(22:1)PC POPC vesicles	55 Å	Fibroblast growth factor receptor (FGFR3)-TM	Donor quenching	13
Cy3	Cy5	POPC vesicles	55 Å	FGFR3-TM	Donor quenching	13
Fluorescein	Rhodamine	POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), and 1-palmitoyl-*sn*-glycero-3-phospho-L-serine (POPS) vesicles	56.1 Å	FGFR3-TM mutants	Donor quenching	55,56
Boron dipyrromethene (BODIPY)-fluorescein	Rhodamine	POPC, POPG, and POPS vesicles	56.3 Å	FGFR3-TM ACH mutant	Donor quenching	12,55,56
Fluorescein	Rhodamine	POPC vesicles	56 Å	FGFR3-TM, Pathogenic Cys mutants	EmEx-FRET Donor quenching	57,58
EYFP	mCherry	CHO cell membrane-derived vesicles	53 Å	Glycophorin A	QI-FRET	59
EYFP	mCherry	CHO, human embryonic kidney 293 cells expressing the SV40 large T antigen (HEK293T) and A431 cell membrane-derived vesicles	Not used	Glycophorin A	QI-FRET	60
EYFP	mCherry	HEK293T cell membrane-derived vesicles	Not used	FGFR3-TM and ACH mutant	QI-FRET	61
EYFP	mCherry	CHO cell membrane-derived vesicles	53 Å	ErbB2 TM and oncogenic mutant	QI-FRET	62
Fluorescein	Rhodamine	Dilauroylphosphatidylcholine (DLPC) and POPC vesicles	55 Å	M2	Donor quenching	63
Fluorescein	Rhodamine	DPC micelles	~50 Å	Major histocompatibility complex chains MHCα and MHCβ	Donor quenching	64

FRET STUDIES OF TM INTERACTIONS IN LIPID AND DETERGENT

Solvent for Membrane Protein Studies: Detergents, Lipids, and Other Lipid Mimetics

Membrane proteins are highly hydrophobic and are generally not soluble in aqueous solutions. Thus, to investigate the structure and thermodynamic properties of these proteins *in vitro*, using any technique, they first need to be reconstituted into environments that mimic natural membranes. Considering the complex constitution of the membrane, it is difficult to replicate the exact conditions for these proteins in an artificial milieu. Nonetheless, tremendous progress has been made to design membrane mimetics that solubilize membrane proteins *in vitro* for their structural and functional characterization (Figure 2).[79,80]

The most widely used membrane mimetics for studying membrane protein oligomerization are lipid bilayers and detergent micelles. Bilayers, which are a more native-like environment, can be synthetically prepared by different methods: in the form of supported bilayers,[76] multilamellar vesicles (MLVs),[13] and giant (~500 nm), large unilamellar vesicles (LUV; ~100 nm), or small unilamellar vesicles (SUV; ~30 to 50 nm). As discussed later, these alternative forms may be best suited to different experimental requirements.[79,80] Another option is the nanodiscs,[81] which are more stable bilayer mimics when compared with liposomes because they are small particles encased by a membrane scaffold protein that surrounds and holds together the lipids. They can be prepared in 6- to 30-nm-diameter sizes based on the scaffolding protein derivative used. Finally, bilayers can also be prepared from natural membranes, such as rough endoplasmic reticulum microsomes[82] and plasma membrane-derived vesicles.[60] These bilayers contain all of the components of a natural membrane.

A bilayer is an ideal membrane mimetic for FRET studies of membrane proteins. However, lipid bilayers are at times difficult or impossible to use because of the challenges with achieving proper equilibration of the samples, along with light scattering artifacts that may affect fluorescence measurements. A less ideal but more convenient alternative is detergents. Like lipids, detergents are amphipathic molecules consisting of a polar head group and a hydrophobic tail; however, instead of bilayers, detergents form globular micellar structures in aqueous solutions. Because of their smaller size, detergents do not produce scattering artifacts and are in general easier to handle. As long as their concentration in solution is above its critical micellar concentration, detergents can generally solubilize a membrane protein and serve as a hydrophobic solvent.
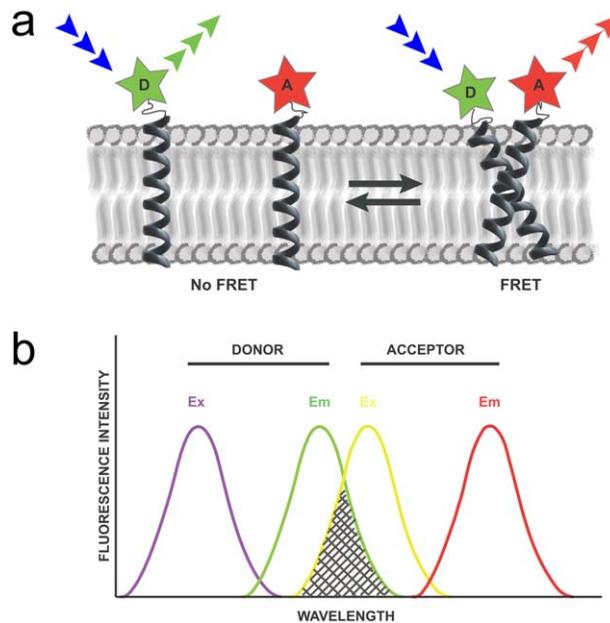

FIGURE 1 FRET as a tool to study transmembrane protein interactions. (a) Schematic representation of TM helices in a lipid bilayer, labeled with donor (D) and acceptor (A) fluorophores. On excitation (blue arrows), the donor fluorophore in isolation emits fluorescence at its characteristic wavelengths (green arrows). In a donor–acceptor dimer, the excitation energy can be transferred from the donor to the acceptor, and FRET will manifest as an increase of acceptor emission (red arrows) and a decrease of donor emission. (b) Depiction of the excitation and emission spectra of a FRET pair. FRET occurs when the emission spectrum of the donor (green curve) overlaps with the excitation spectrum of the acceptor (yellow curve) with a good overlap integral denoted by the gray shaded area. A good FRET pair has a notable spectral overlap with minimal bleed-through of the acceptor's excitation into the donor excitation wavelength (overlap of the violet and yellow spectra) and minimal emission of the donor in the acceptor emission wavelength (overlap of the green and red spectra). Colors of the spectrum are a depiction of the increasing wavelength of light.

A vast array of detergents with distinct physical–chemical properties can be chosen from to optimize conditions for a specific protein and application.[80,83–85] Although detergents can conveniently solubilize membrane proteins, they can also destabilize the proteins over time. In an effort to design less destabilizing solvents, compounds called tripod amphiphiles[86,87] and amphipols[69,88] have been developed and used for solubilizing membrane proteins. These compounds are designed to keep membrane proteins active over periods of time and have been useful in structural investigations using NMR and X-ray crystallography. In certain situations, a combination of detergents and lipids to form mixed micelles[45] and bicelles[89] (small discoidal lipid bilayers whose edges are stabilized by short-chain lipids) can be used and has been useful in many structural investigations of membrane proteins.
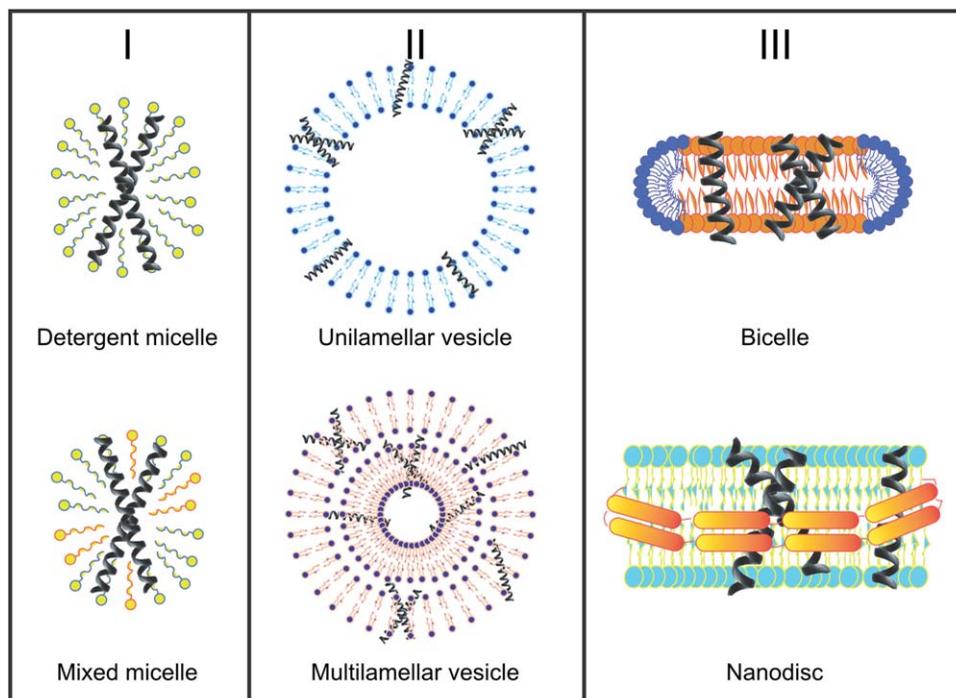
FIGURE 2 Artificial environments for membrane protein solubilization. (I) Transmembrane helices can be incorporated into micelles made of a single detergent (top) or by mixtures of different detergents and lipids to form mixed micelles (bottom). (II) Lipid bilayers in the form of unilamellar (top) or multilamellar (vesicles). (III) Two types of discoidal bilayers, bicelles, which are bilayers stabilized by short-chain lipids at their edges (top) and nanodiscs, stabilized by membrane scaffolding proteins (bottom). The figures are not drawn to scale.

Hydrophobic Environment Properties Affect Helix–Helix Associations

Investigations of TM peptides in detergents and lipids have enabled identifying key residues involved in helix–helix associations and contributed greatly to our understanding of the factors that influence the energetics of association in membrane proteins. The TM domain of GpA (GpA-TM) has been a major paradigm in such studies, and the very first systems investigated using FRET in a pioneering investigation that used dansyl- and dabsyl-labeled peptides in dimyristoylphosphatidylcholine (DMPC) MLVs[26] (the study was more precisely designated as "RET" because the acceptor dabsyl is nonfluorescent). Numerous studies in lipids and detergents have followed this initial work to better understand the thermodynamics of GpA-TM dimerization as well as the environmental influences on membrane protein interactions. For example, FRET was used to investigate the effect of different types of detergents on the GpA-TM associations.[11,44] The GpA-TM dimer was observed to be two orders of magnitude less stable in sodium dodecyl sulfate (SDS) than in zwitterionic detergents *N*-dodecyl-*N,N*-(dimethylammonio)butyrate

(DDMAB) and dodecylphosphocholine (DPC), demonstrating that environment has an important role to play in association.[11] The time taken to reach steady-state equilibrium also depended on the type and concentration of the detergents used. A follow-up paper expanded on the previous study by studying GpA-TM dimerization in a series of detergents with varying alkyl chain lengths, combined with ionic, nonionic, and zwitterionic headgroups.[44] The results suggested that dimerization of GpA-TM in detergents is influenced by two opposing effects: an enthalpic effect, which drives association with increasing detergent concentration and which is sensitive to the detergent headgroup chemistry, opposed by an entropic effect, which drives peptide dissociation with increasing detergent concentration. Thus, this study begun shedding light on the importance of detergent selection and how they can influence the association equilibrium of helical dimers.

Although these studies focused on the effect of detergents with different head group chemistry, a clear relationship between the alkyl chain length and the strength of dimerization was not observed. A much later study investigated the effect of aggregation number, or the number of detergent

monomers per micelle, on TM helix dimerization. FRET measurements using labeled GpA-TM peptides were carried out on lyso-PC micelles of varying acyl chain lengths to investigate the effect of detergent concentration, aggregation number, and the micelle's hydrodynamic radius on TM association.[70] It was observed that the dissociation of the GpA-TM dimer did not correlate to dilution in detergent, indicating an influence of the detergent properties on dimerization. Furthermore, GpA-TM dimerization did not depend on the similarity of the detergent's hydrodynamic radius to the hydrophobic length of the TM domain; in fact, the oligomerization was destabilized with increasing chain length, opposing the assumption that longer chains render the detergents milder. Moreover, the detergent molecules in a micelle seemed to be able to match the hydrophobic length of the peptide, suggesting that the concept of hydrophobic match/mismatch of TM helices to lipid membrane systems[90] may not be translated to detergent systems. On the other hand, the effect of acyl chain length of lipid vesicles had a direct correlation with GpA dimerization in different lipid vesicles with varying carbon chain lengths.[54] Unlike the observation for the lyso-PC detergents, the acyl chain length was found to severely influence the dimerization of the TM domain, being most efficient under hydrophobic length-matching conditions. Dimerization efficiency was highest in the C20 (30.5 Å) and C22 (34 Å) chain length lipids comparable with the hydrophobic length of GpA (31–32.2 Å), according to the NMR structure of the TM dimer[91] and the theoretical prediction of the OPM database[92]. This, again, demonstrated how different environments play a role in the association of a TM protein.

FRET Used to Study "Unfolding" of TM Proteins

To study reversible folding of membrane protein systems, conditions are required that push the equilibrium to the dissociated state. This can be achieved by diluting peptide concentration in the hydrophobic volume as performed in typical FRET experiments. Apart from dilution in the same detergent or lipid, unfolding studies of TM helix association using FRET have also been attempted by changing the hydrophobic environment from a more stable to a less stable state. A first report of the utility of mixed micelles using GpA as a dimerization standard[45] provided evidence that unfolded/unassociated membrane proteins retain a majority of their helical content, as postulated by the Two-Stage Model of membrane protein folding.[8] Labeled GpA TM peptides were reconstituted in the mild nonionic dodecylmaltoside (DDM) micelles, where they were helical as well as dimeric as observed by FRET. Keeping the total peptide:detergent ratio constant, SDS was added such that the mole fraction of SDS relative to

DDM increased. SDS is a harsh, negatively charged detergent shown to denature most polytopic membrane proteins; however, it retains the oligomeric states of some TM helices in the low millimolar concentrations.[18,93–96] It was observed that with increasing mole fraction of SDS, there was a decrease in FRET but not helicity of the sample (observed by CD), indicating dissociation of the dimer to form helical monomers in mixed micelles.[45]

In a similar unfolding study, two TM helices of bacteriorhodopsin that are known to associate (helices A and B) were transferred from 1,2-dioleoylphosphatidylcholine (DOPC) bilayers to SDS micelles.[46] This study involved the use of intrinsic FRET from a tyrosine on one peptide to two tryptophan residues on the other peptide. FRET efficiency was reduced from transferring the peptides from DOPC to SDS micelles, suggesting a loss of association, interpreted as unfolding of the peptides from DOPC bilayers to SDS micelles. However, MD simulations in the same study suggested that a small rearrangement of the peptide backbone in the SDS micelles could result in easier access of water molecules into the micelles. This water could form hydrogen bonds with the donor (Tyr), which could decrease the quantum yield and contribute to a decrease in the FRET efficiency.

The authors also argued that the use of mixed micelles in TM helix unfolding may be overlooking the effects of increased surface charge density of the negatively charged SDS micelles,[45] which may affect the properties of dyes such as fluorescein and 5-carboxytetramethylrhodamine, which are environmentally sensitive.[97] This study highlights one of the complexities of using FRET to study membrane proteins where changes in the hydrophobic environment can affect the chromophores and to contribute a change in the FRET efficiency. In such situations, control experiments using different fluorophores or with alternative biophysical methods could help to recognize the influence of these undesired environmental effects.

Comparing Stability in Lipids and in Detergents Using FRET

As the lipid bilayer is closer to the natural membrane in comparison with detergent, it is expectable that the oligomeric complexes may be better behaved and more stable in the former. This distinction has been demonstrated using FRET in different systems. The oligomeric state of phospholamban (PLB), a 52-amino acid protein in the cardiac sarcoplasmic reticulum that regulates the cardiac calcium pump, was investigated both in detergent and in lipid bilayers.[28,98] It was previously shown that PLB forms homopentamers in SDS micelles.[99,100] An electron plasmon resonance (EPR) study[101] showed that the average oligomeric

size of PLB changed from 3.5 to 5.3 on its phoshorylation, suggesting a dynamic equilibrium between PLB subunits in lipid bilayers. This suggested that there might be oligomeric changes involved in the regulation of the pump; however, the EPR method could not resolve the multiple oligomeric species. Using fluorescent donors such as 6-[(6-amino-4-methylcoumarin-3-acetyl)amino] hexanoic acid (AMCA) and 5-dimethylamino-1-naphthalenesulfonyl (DANS) and a nonfluorescent 4-(dimethylamino)azobenzene-4-sulfonyl (DABS) acceptor attached to recombinant PLB protein, Li et al.[28] analyzed the PLB oligomeric states in detergents and lipids. The various detergents yielded to different observations in terms of the extent of oligomerization, the oligomeric state, and the proximity of adjacent subunits. Moreover, a marked difference in sample equilibration was observed between lipid (DOPC) and the detergents. Equilibration occurred readily in lipid, whereas a boiling step was required to observe efficient subunit exchange between the complexes in detergents. Once again, these observations demonstrate how the environment can play a role in the association of TM proteins.

Other FRET studies of oligomerization have demonstrated that TM helix interactions can be more stable in the lipid bilayer. FRET efficiency values indicated that the dimerization of the TM domain of the EphA1 receptor was more stable in DOPC bilayers than in SDS.[47] Similarly, the formation of homooligomers and heterooligomers of the bacterial divisome proteins FtsB and FtsL demonstrated that all complexes were more stable in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles than DPC micelles.[14] The weaker oligomerizing tendency in DPC of the FtsB TM peptide (which has previously been shown to self-associate in native bacterial membranes[102] suggested that the contribution of a hydrogen bond formed by a glutamine residue at the FtsB interface may be weakened by the increased ability of this polar residue to interact with water molecules in the micellar environment.

FRET in Detergent as a Complementary Assay

Despite the higher stability of TM peptide oligomers in lipid, detergents pose a very convenient means for these experiments. FRET in detergent micelles has aided the study of the interaction of many TM helix systems. These studies have often been performed in coordination with other methods such as coimmunoprecipitation, PAGE, Western blot, TOXCAT,[103] and SE-AUC to study a variety of membrane protein systems. For example, Deber and coworkers[49] have associated FRET with the aforementioned techniques to study the homodimerization of the TM domain of the major coat protein of Ff bacteriophage in SDS micelles; the oligomerization of the Na,K-ATPase

(sodium pump) TM domain in perfluorooctanoate (PFO) micelles (a detergent that retains association for weakly interacting helices)[51]; and the TM self-association of the anthrax toxin receptor ANTRX1 in SDS micelles.[50] In addition to these biological systems, they also studied a model helix–loop–helix peptide made of Ala and Ile residues in SDS micelles to investigate the nature of van der Waals forces and side-chain hydrogen bonding networks in TM peptide interactions.[53]

FRET in detergent micelles has also aided computational design. An example is the experimental characterization of the association of computed helical antimembrane protein (CHAMPs) peptides to their target, which are capable of binding specifically to TM helices in a similar fashion to antibodies of water-soluble proteins.[52] The fluorophores and experimental conditions used in all the above-mentioned studies are listed in Table I.

Choices of Lipid Environments: Artificial Bilayers

Different forms of artificial lipid bilayers are suitable for FRET studies of TM helix interaction: MLVs or unilamellar vesicles of varying sizes and planar-supported bilayers. The MLVs are prepared by hydration, vigorous agitation, and freeze–thawing of hydrated peptide/lipid films, which are obtained by evaporation from organic solutions.[13–15] It was observed that the presence of TM peptides decreases the turbidity of MLVs after just one freeze–thaw cycle. This change was not observed for free lipids,[13] indicating that the presence of peptides facilitates the formation of smaller MLVs in the 1:100 to 1:10,000 peptide:lipid relative molar range, making FRET experiments in these vesicles feasible. Extrusion of MLVs using a 100-nm-diameter membrane (Avanti) produces LUVs. Sonication of the extruded LUVs creates 30- to 50-nm SUVs. Although the fluorescence of the TM peptides tends to be stable over a long period of time in MLVs and LUVs, it decreases more rapidly in SUVs indicating a higher tendency of aggregation of the TM peptide.

In theory, the unilamellar vesicles have the advantage to rule out any spurious FRET contributions arising from TM peptides in consecutive leaflets of an MLV. It has been shown, however, that there is no statistically significant difference between FRET efficiencies obtained from MLVs and LUVs. Extrusion can cause a loss of peptide as well as lipid; however, this loss is to a similar extent in both, and thus does not affect the FRET efficiency (which is a function of the peptide:lipid ratio).[13] Among these choices, the MLVs are the most practical for their stability and short preparation time, and thus represent a good hydrophobic environment to investigate TM helix–helix interactions for bulk FRET studies.[13,14]

Another synthetic bilayer platform developed and extensively used by Hristova and coworkers is the surface-supported

bilayer, where peptides are reconstituted into a single leaflet of lipid at the air/water interface, transferred onto glass substrates, then fused with lipid vesicles to form the second leaflet of the supported bilayer, and imaged under a microscope.[73–75] This "directed assembly" of the peptides into the bilayers is different from the previously designed "self-assembly" of peptide-containing liposomes on glass surface[104] and offers several advantages over it. First, it requires 1/100th the amount of peptide needed for reconstituting in bilayer vesicles in solution before assembling them on the glass surface, thereby reducing the material cost of the experiments. Second, it allows more control over achieving unidirectional orientation of the peptides. The peptides are deposited on a single lipid monolayer first and can be oriented according to a certain topology by ensuring that the TM region is flanked on one side by charged water-soluble residues and on the other side by neutral hydrophilic residues.[73]

Instrumentation advances have also contributed to expanding the range of conditions in which these supported bilayers can be applied to measuring association of membrane proteins. Li and Hristova[104] initially developed the techniques relying on fluorescence recovery after photobleaching (FRAP) using acceptor photobleaching as the observed parameter. Fluorescence was measured using a microscope (imaging FRET). The method was later adapted to allow the measurement of fluorescence in supported bilayers using a standard spectrofluorimeter (spectral FRET).[74] The use of "spectral FRET" technique does not require the stringent conditions imposed by FRAP, which necessitates that the peptides in the bilayer diffuse slowly and that the fluorophores are photostable. Moreover, detection with a spectrofluorimeter allows one to record the entire spectra, as opposed to fluorescence intensity within a narrow wavelength range determined by the choice of emission filters, as in "imaging FRET." These developments enabled a wider selection of FRET pairs (as reported in Table I), including pairs such as fluorescein/rhodamine that have greater spectral bleed-through and lower photostability, but are more commonly used for their ease of labeling, cost effectiveness, and favorable Förster radius for TM helix interactions.[74,105] Hristova and coworkers have integrated the use of all the types of bilayers described above—LUVs, MLVs, and surface-supported bilayers—to study TM interactions of the receptor tyrosine kinase (RTK) FGFR3 and its sequence variants.[12,42,55–58,106]

Choices of Lipid Environments: Plasma Membrane-Derived Vesicles

Although artificial bilayers have enabled thorough investigations of membrane protein association energetics using FRET,

one caveat is that the interactions are assayed in a non-native "dilute" system. In the cell membrane, protein–protein interactions occur in a crowded environment, with a high local concentration of other proteins. *In vitro* experiments with artificial bilayers ignore the "excluded volume effect" described for soluble proteins, which explains how macromolecules inside a cell occupy 20–30% of the cell volume, exerting nonspecific effects on the interactions.[107,108] This type of crowding also occurs in the membrane with other proteins in the vicinity and could either increase the probability of association or prevent specific associations of membrane proteins in the local membrane environment. This supports an argument that working in artificially dilute conditions, for any macromolecular interaction study, is associated with a risk of obtaining results that are physiologically irrelevant.[109]

To account for this crowding factor, Hristova and coworkers[72] developed a quantitative FRET imaging technique (QI-FRET), which used microscopy to measure TM interactions in cell-derived vesicles that contain the native cytoplasmic milieu. Using this technique, they found that the GpA-TM dimer is in fact weaker in mammalian membranes[77] than previously established using various techniques,[11,18,26,44,103,110–113] suggesting that molecular crowding indeed plays a role in the energetics of protein–protein interactions. They further established that the dimerization of GpA-TM is not statistically different in a variety of six different plasma membrane-derived vesicles prepared from different cell lines using different methods,[60] suggesting that any of these cell lines could be used for TM domain interaction studies using QI-FRET. QI-FRET was also applied to investigate oligomerization of members of the RTK family oligomerization. Among the findings, it was determined that a disease-causing mutation in the TM domain of FGFR3 has altered dimerization[61] and that the ErbB2 TM domain shows the highest dimerization strength amongst the RTKs in mammalian membranes.[62] A list of the various mammalian membranes used in these studies is provided in Table I.

CALCULATION OF ENERGETICS OF ASSOCIATION FROM FRET EFFICIENCY

The FRET efficiency (E) is the fraction of energy from photons absorbed by the donor that is transferred to the acceptor.[114] Energy transfer occurs only when two fluorophores are at a distance that is near or below the characteristic Förster radius. As such, the FRET efficiency can be used as a reporter of intermolecular interaction.

In the simplest case of a dimeric complex, the amount of observed FRET E_{obs} is theoretically directly proportional to the relative amount of dimer complex according to the following formula equation:

$$\frac{E_{\text{obs}}}{E_{\text{max}}} = \frac{2[d]}{2[d]+[m]}, \qquad (3)$$

where $[d]$ is the concentration of the dimer, $[m]$ is the concentration of the monomer, and E_{max} is the maximum theoretical FRET efficiency. The monomer and dimer fractions can be then used to derive the apparent equilibrium constant and free energy of association.

$$K_{\text{D}} = \frac{[m]^2}{[d]}, \qquad (4)$$

$$\Delta G^0 = -RT\ln(K_{\text{D}}). \qquad (5)$$

The maximum theoretical FRET efficiency, E_{max}, is the FRET of a sample in which the equilibrium is completely pushed toward the dimeric form. E_{max} depends on the relative concentration of dimer formed by a donor (D) and an acceptor (A) peptide, over all other possible combinations of dimers that involve a donor. E_{max} also depends on the presence of any unlabeled peptide (U), which will also participate in the equilibrium competing with the observable donor–acceptor pair.

$$E_{\text{max}} = \frac{[DA]}{[DA]+[DD]+[DU]}. \qquad (6)$$

The concentration of unlabeled peptide depends on the labeling efficiencies of donor and acceptor samples. The maximization of labeling efficiency is a critical factor for a successful FRET experiment. As discussed later, the equilibrium models should also take into account, any contribution to FRET by peptides that colocalize randomly due to diffusion but are not engaged in a specific complex (proximity FRET).

The Simple Formalisms

The basic measurement of FRET efficiency requires at least three different samples for every peptide concentration used; the "donor-only" sample, the "acceptor-only" sample, and the "FRET sample," containing a mixture of donor- and acceptor-labeled peptides (Figure 3). Primarily, three different techniques have been used to measure the FRET efficiency of TM helix–helix oligomerizations.

The first technique is donor quenching. This approach relies on the decrease of the direct emission of the donor, that results from the energy transfer to the acceptor. Using the donor-only and the FRET sample, the apparent FRET efficiency E_{app} is calculated from the intensity of donor emission when acceptor is present (I_{DA}) and when it is absent (I_{D}) as follows:

$$E_{\text{app}} = \frac{(I_D - I_{DA})}{I_D}. \qquad (7)$$

The fluorescence intensity is generally measured at the emission maximum wavelength λ_{max} of the donor (Figure 4).

The second method is acceptor sensitization, which measures the increase in acceptor emission, or "sensitization." As in most cases there is some direct excitation of the acceptor (I_{A}) when excited at the donor excitation wavelength (Figure 4), this contribution needs to be subtracted from the acceptor emission (I_{AD}) measured from the FRET sample. The apparent FRET efficiency is then given by the following equation:

$$E_{\text{app}} = \left(\frac{(I_{AD}-I_A)}{I_A}\right)\frac{\varepsilon_A}{\varepsilon_D}, \qquad (8)$$

where ε_{D} and ε_{A} are the molar extinction coefficients of the donor and the acceptor at the donor's excitation wavelength.[115] The acceptor sensitization method is most useful when using a FRET pair such as pyrene (Pyr) and coumarin. Pyr has a high quantum yield and narrow bands (Figure 5a), making it easier to detect energy transfer through sensitized acceptor emission. In Figure 5c, the emission detected at 500 nm is plotted as a function of excitation wavelength. The intensity due to direct emission of the donor is minimal, and a large increase in the excitation intensity between 300 and 350 nm indicates a strong FRET signal. Another advantage of the Pyr/coumarin pair is that the presence of the donor affects neither the relative fluorescence nor the wavelength of the acceptor maximum (378 nm), allowing it to be used as an internal standard for acceptor concentration during the FRET measurements.

One of the uncertainties associated with FRET measurements is the variation in the effective peptide concentration from one sample to another, which is a particularly important issue working with hydrophobic TM peptides. The EmEx-FRET technique reduces these uncertainties by calculating the concentrations of the donor- and acceptor-labeled peptides from the fluorescence spectra obtained.[57] In this technique, the excitation and the emission spectra for the FRET sample are obtained and normalized to the excitation and emission spectra of donor-only and acceptor-only samples of known concentrations (standards) using a scaling coefficient factor. This coefficient factor is multiplied by the known standard concentrations to get the donor and acceptor concentrations in the FRET sample.[57,105]

Which Method Should Be Used to Calculate FRET Efficiency?

The most commonly used method for membrane proteins is donor quenching, as is reflected in Table I. Although acceptor
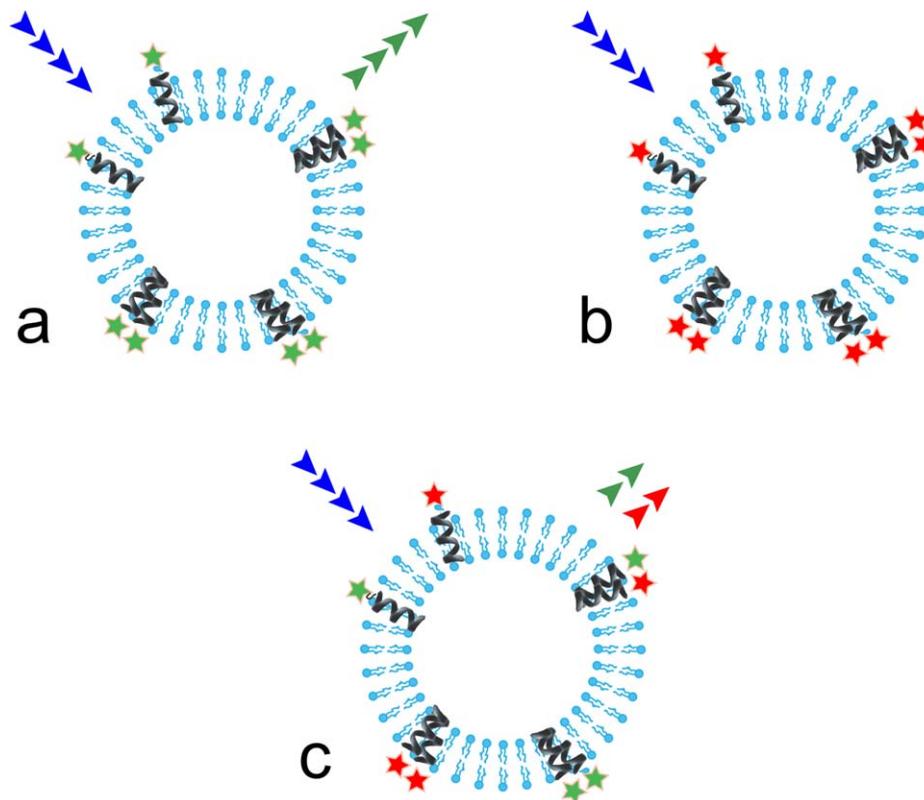
FIGURE 3 Control samples in a FRET experiment. (a) "Donor-only" sample: Peptides labeled with donor fluorophores are excited with donor excitation wavelength (blue arrows) and exhibit donor fluorescence (green arrows). (b) "Acceptor-only" sample: Peptides labeled with acceptor fluorophores are excited with donor excitation wavelength and show no red fluorescence (minimal spectral bleed-through). (c) "FRET sample": Peptides with equimolar donor:acceptor peptide ratio, with the same total peptide:lipid ratio, excited with donor excitation wavelength exhibit acceptor fluorescence (red arrows) due to FRET arising from TM peptide association and show decreased donor fluorescence (green arrows).

sensitization provides a more direct measurement, it requires characterization of the acceptor's extinction coefficient in the detergent or lipid being used, which is nontrivial for less photostable environmentally sensitive dyes.

The EmEx method of FRET provides a more precise measurement by actually measuring the effective concentration of both the donor- and acceptor-labeled peptides during the fluorescence acquisition. This method requires the FRET pair to have a wavelength range where the acceptor's excitation spectrum in the presence and absence of the donor is the same, a feature of common pairs such as fluorescein–rhodamine and Cy3–Cy5 and also fluorescent proteins such as enhanced yellow fluorescent protein–mCherry. Moreover, the EmEx concept, which has been adapted for cell-derived vesicles as the quantitative imaging of QI-FRET,[72] can also be used in cellular studies where the donor and acceptor concentrations are unknown.

This technique was developed using the FGFR3-TM peptides in POPC vesicles,[57] and subsequently extended to FRET microscopy to yield the quantitative imaging or QI-FRET method that allows the determination of the donor and acceptor concentrations as well as FRET efficiency in cell-derived vesicles.[60,61,72,77] However, the QI-FRET imaging technique measures the integrated intensity of fluorescence over a particular bandwidth of an optical filter, and not the full spectrum as opposed to that obtained from a fluorimeter. Therefore, although this method provides the most physiologically relevant measurement of FRET efficiency of membrane proteins, adaptation of this method to obtain fluorescence spectra instead of integrated intensities may provide a more quantitative analysis of the interaction energies of association.

In summary, the choice of method used therefore depends on the nature of the investigation and may be narrowed down
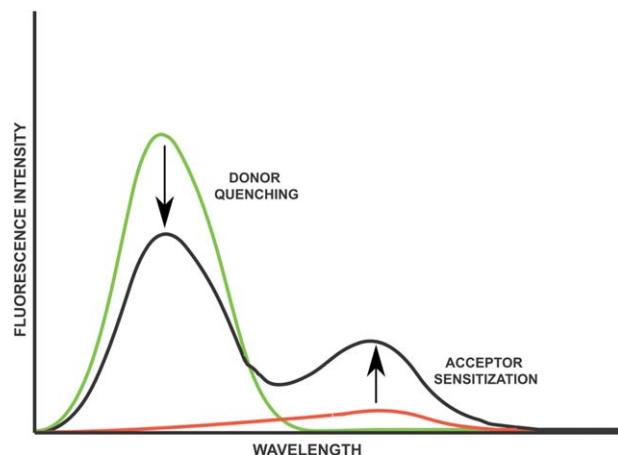
FIGURE 4 Calculation of the FRET efficiency. The green curve represents a fluorescence spectrum of the "donor-only" sample, representing the donor emission spectrum at the donor's excitation wavelength. The red curve represents a spectrum of the "acceptor-only" sample from direct excitation of the acceptor at the donor's excitation wavelength due to spectral bleed-through. The black curve represents an emission spectrum of the "FRET sample" containing a mixture of donor and acceptor, excited at the donor's excitation wavelength. FRET efficiency can be calculated either by monitoring the decrease in the donor's emission intensity (indicated by downward arrow) or by monitoring the increase in the acceptor's emission intensity (indicated by the upward arrow).

FIGURE 5 Demonstration of sensitized emission using pyrene- and coumarin-labeled peptides. The fluorescence spectra shown are that of glycophorin A peptides labeled with pyrene (donor) and coumarin (acceptor) in DDMAB detergent. (a) Pyr-GpA: Excitation spectra (—) recorded at 378 nm emission wavelength, and emission spectra (– – –) recorded at 345 nm excitation wavelength. (b) Cou-GpA: Excitation spectra (—) at 460 nm emission wavelength and emission spectra (– – –) at 378 nm excitation wavelength. (c) Excitation spectrum of a "donor-only" sample (- - -), an "acceptor-only" sample (– – –), and a 1:1 donor:acceptor "FRET sample" (—) recorded a 500 nm emission wavelength. Sensitized emission was observed by an increased intensity of the Pyr-GpA (donor) excitation spectrum in the "FRET sample" when compared with that in the "donor-only" sample. Cou-GpA (acceptor) spectrum was unaltered between the "acceptor-only" and the "FRET sample." (Reproduced from Ref. [11], with permission from Elsevier.)

to donor quenching, the EmEx, and the QI-FRET methods as the most practical choices. For qualitative applications, the donor quenching method is straightforward, and the fastest, and can also be used for quantitative analyses along with the use of proper controls.[13] For a more rigorous study of the energetics of TM helix association, the EmEx method serves as a precise technique that can provide measurements in artificial bilayers in a fluorimeter and can be used with a wider array of FRET pairs to choose from, and the QI-FRET method extends this analysis to proteins in native mammalian cell-derived vesicles with the help of confocal microscopy.

Nonspecific Proximity Contribution to FRET

For a thorough quantification of FRET efficiency, another important parameter is the contribution due to random colocalization rather than association. Studies of interactions between TM helices are usually performed in sufficiently dilute conditions (i.e., low peptide:lipid ratios) so that the average distance between individual peptide molecules is greater than the Förster radii of the dyes they are attached to. Nevertheless, free diffusion of the peptides will allow an extent of random colocalization of donor and acceptor molecules that cohabitate the same lipid vesicle. Thus, the
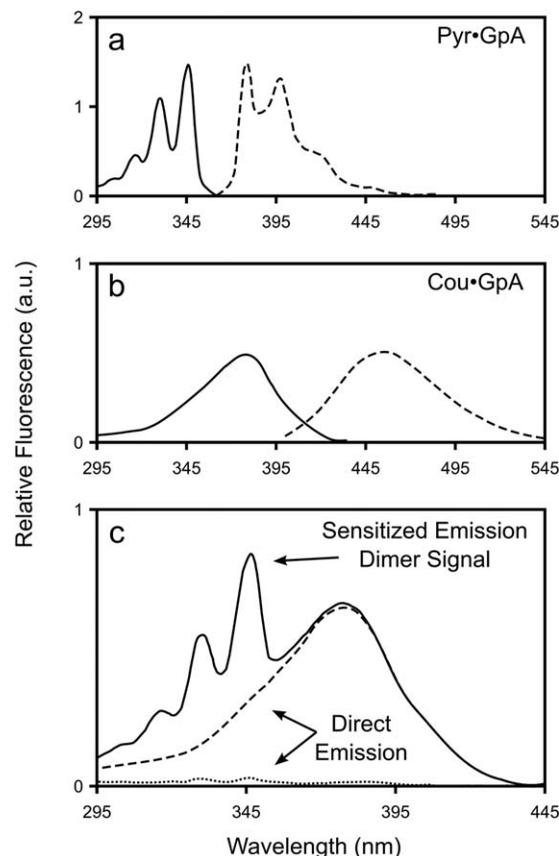
observed FRET (E_{obs}) will contain a contribution of proximity FRET ($E_{\text{proximity}}$).

$$E_{\text{obs}} = E_{\text{interaction}} + E_{\text{proximity}}. \qquad (9)$$

The FRET due to random proximity was not included in the earlier quantitative models.[25,26,28] This contribution factor was first simulated for randomly distributed peptides, whose donor quenching by different acceptors in various acceptor configurations was averaged for different Förster radii.[65,66] The proximity term is given by the following equation:

$$E_{\text{proximity}} = \frac{1}{1 + \sum_i (r_i/R_0)^6}, \qquad (10)$$

where r_i is the distance between the donor and the ith acceptor, and R_0 is the Förster radius for the FRET pair. This proximity FRET term was then used by Hristova and coworkers[13] to separate the signal due to sequence-specific interaction from random colocalization in lipid bilayers. The application of this factor demonstrated that there is a marked contribution of proximity to the observed FRET, with higher contribution for FRET pairs having higher R_0 values, even at low acceptor mole fractions.[13] It was also observed that FRET due to donors and acceptors between two consecutive leaflets of an MLV is relatively small, even for higher R_0 values, confirming that the use of MLVs does not increase substantially the contribution of proximity FRET.

These proximity simulations considered the organic fluorophores to be point structures negligible in size, thus they are not applicable to large (3–4 nm) fluorescent proteins, which have a limited distance of closest approach. A model that did take the size factor into account developed the proximity contributions for monomeric fluorophores of a finite size,[116] but not for those attached to oligomeric membrane protein complexes.

For cellular FRET studies, the fluorophores primarily used are large fluorescent proteins (FPs). These fluorophores participate in oligomeric structures where the probability of "background FRET"[117] from a "bystander protein"[118] in a crowded environment is quite high. King et al.[67] predicted the proximity FRET over a wide range of exclusion radii of fluorophores and a thorough range of acceptor concentrations. They also simulated the proximity FRET for monomers (which they verified experimentally using plasma membrane-derived vesicles), dimers, trimers, and tetramers for the first time and generated a model for the proximity FRET contribution as a function of oligomeric state and geometry of membrane protein complexes in a cellular scenario.

A different formalism of separating the proximity and specific interaction terms, argued to be more accurate,[34] has been used as well where both the FRET and proximity contributions are multiplied and integrated to obtain the donor fluorescence decay, instead of the proximity contributions being subtracted while using the steady-state donor quenching.[68]

Beyond the quantification of the contribution of FRET due to specific interactions, a practical control to determine whether the observed FRET is primarily due to sequence-specific interactions is the addition of unlabeled peptides to the FRET sample, which should result in a decrease in FRET by competition.[13,14] For instance, in the case of a dimeric interac-

tion, if the addition of an equimolar amount of unlabeled peptide results in a FRET decrease of ∼50%, it would indicate that the observed FRET is not majorly affected by unwanted effects such as colocalization, donor self-quenching, improper equilibration, or interference from labels.[11,53] A useful additional control is the addition of the same amount of an unrelated peptide, which theoretically should not result in significant change of FRET. (Note: The actual theoretical decrease in these competition experiments depends on the total peptide concentration and the dissociation constant, because the addition of unlabeled peptide increases total peptide concentration.)

Choice of Fluorophores

An important parameter affecting the observed FRET efficiency is the nature of the fluorophores involved. An ideal FRET pair is one that has good spectral overlap, high photostability, high dynamic range, and minimal spectral cross-talk. One of the first decisions is whether to prepare TM domain fusion constructs with fluorescent proteins or to chemically synthesize the TM domains and label them with small fluorophores. Genetically encoded fusions with fluorescent proteins (FP) are faster and more cost effective than synthesizing peptides and labeling them with small fluorophores; they also pose the advantage of conferring solubility to the hydrophobic TM domain during overexpression and purification. However, as can be seen in Table I, the use of FP fusion is not common unless the study is performed in cell membrane-derived vesicles because of the low FRET dynamic range, poor maturation, and reversible and irreversible photobleaching of FPs. Although efforts to improve on the most standard FP FRET pair (CFP and YFP) are constantly ongoing,[119,120] the use of organic fluorophores has been the most common in *in vitro* studies on TM proteins.

Fluorescein is a widely used fluorophore more commonly as a donor, such as in the fluorescein–rhodamine pair, but also as an acceptor, as in the coumarin–fluorescein pair. With an R_0 value of ∼55 Å, the fluorescein–rhodamine FRET pair is desirable because it can yield a FRET efficiency of up to ∼99% for a typical parallel helical bundle, which has a much smaller interhelical distance of 10–20 Å. As a donor, fluorescein can have a problem of self-quenching because of its overlapping excitation and emission spectra; however, control experiments can be carried out to rule out that contribution as shown by You et al.[13] Fluorescein is used as an acceptor in the coumarin–fluorescein pair. Its Förster radius, however, has not been well characterized in detergent or lipid, resulting in uncertainty regarding the maximum FRET efficiency that can be observed. Nevertheless, this FRET pair has been used in applications, with the use of donor quenching to calculate FRET efficiency.[14,52]

An alternative fluorophore that is more photostable and does not exhibit self-quenching due to overlapping excitation and emission spectra is Cy3, which forms a FRET pair with Cy5 with a comparable R_0 value with fluorescein–rhodamine. However, as can be seen in Table I, this FRET pair is not very widely used for membrane protein experiments. The low adoption of this pair is mainly due to cost. The labeling of hydrophobic TM peptides is difficult and requires large excess of dyes (∼12 equivalents) to achieve maximum labeling efficiency,[14,71] which can render the use of the costly Cy3 and Cy5 dyes prohibitively expensive.

Other FRET pairs such as Pyr–coumarin[11,44] and DANS–DABS[26,28,49,50,53] have also been commonly used and have served as good FRET pairs for membrane protein interaction studies.

DETERMINATION OF THE OLIGOMERIC STATE USING FRET

FRET is most suited to investigations of pairwise donor–acceptor interactions, nevertheless a number of models have been developed to use FRET for the determination of the oligomeric state of TM complexes.

Using the fluorescence quantum yield of the donor as a function of the mole fraction of acceptor (keeping the total peptide:lipid ratio constant), Veatch and Stryer[25] were the first ones to postulate the different signature curves for different oligomeric schemes (dimer, trimer, and tetramer) of membrane proteins in lipid. They also demonstrated how the equilibrium constant can be determined from the characteristic quenching curve of the donor as a function of the acceptor mole fraction once the number of subunits has been defined, and how the slope of the curve can be correlated to a preference for either donor–donor and acceptor–acceptor (homo-pairs) pairs over the productive donor–acceptor FRET sample (hetero-pair). However, although the FRET data best supported a dimeric state for gramicidin A TM channel in dihexanoylphosphatidyl-choline (DHPC) vesicles, complementary conductance measurements were necessary to rule out possible trimer and tetramer models, which exemplify the difficulties in assessing the size of an oligomer by FRET alone.

In 1994, Adair and Engelman[26] derived a dependency of the donor quenching on the acceptor mole fraction, showing that a linear relationship between the two was consistent with a dimer model for GpA in DMPC vesicles. Working under assumptions that there was no preference between homo-pairs, hetero-pairs, and pairs with unlabeled peptides, they demonstrated that the data had a better fit to a dimer model (0.97 R^2) as opposed to that of a trimer (0.68 R^2). This relationship has since been widely used to elucidate the oligomeric state of many membrane protein complexes in lipid and detergent.[47–49,53,54,78] It is important to note that oligomeric state predictions obtained by these FRET experiments were, in all cases, complemented by investigations with other techniques. For example, the nonlinear dependency of FRET on the acceptor mole fraction observed for the IRE1 TM domain fit well to a tetramer model; however, this result was further corroborated by SDS-PAGE analysis, which displayed a band with an apparent molecular weight compatible with such an oligomer.[48]

The formalisms of Veatch and Stryer[25] and Adair and Engelman,[26] which work under the approximation of equal energy transfer to all subunits in an oligomer, are theoretically applicable to elucidating higher-order oligomeric states. However, in the literature, they have been applied primarily to dimerizing complexes.[47,49,53,54,78] This is likely because the linear relationship between FRET efficiency and the mole fraction of the acceptor holds only in the dimer case, and because the shift between the theoretical curves is much more marked in the dimer–trimer transition than in the transitions between trimer–tetramer, tetramer–pentamer, and so forth.

A more complex formalism that in principle accounts for the contribution of all subunits, taking into account the geometry of a higher-order oligomeric complex, was developed by Thomas and coworkers.[28] This model, which does not assume equal energy transfer between all subunits, was applied to PLB, a regulator of the Ca^{2+} cardiac pump. The FRET efficiency of each donor subunit to each acceptor subunit was calculated explicitly, assuming a ring-like structure, and the dependency of FRET on acceptor mole fraction was simulated for 2–11 subunits. The data showed that in DOPC, PLB was in dynamic equilibrium, and the analysis indicated that the predominant forms had 8–11 subunits, in disagreement with the physiological pentameric form. This work exemplifies the difficulties in differentiating between higher-order oligomeric states using FRET due to uncertainties in peptide concentration, labeling efficiency, and the position, orientation, and mobility of the fluorophore as well as effects of the local environment on their quantum yield, all of which can severely affect the modeling. Nevertheless, such theoretical models can be applicable to cases in which the oligomeric state is known, such as an analysis of the relative stability of the M2 channel tetramer in different bilayers.[63]

FRET can also be used to obtain the stoichiometry of a heteromeric complex. This has been demonstrated by different experiments involving donor- and acceptor-labeled peptides in fixed donor:acceptor and fixed peptide:lipid/detergent ratios and performing competition experiments on those samples using unlabeled peptides. For example, the stoichiometry of the heterodimeric Class II major histocompatibility complex

(MHC) chains, MHC donor and MHC acceptor TM peptides, was found to be 1:1 by competing with unlabeled MHC TM peptides.[64]

Similarly, using a series of FRET experiments, Khadria and Senes[14] were able to determine that the bacterial cell division proteins FtsB and FtsL form a 1:1 higher-order oligomer (hetero–tetramer or even higher). First, it was demonstrated that FtsB and FtsL interact strongly using donor-labeled FtsB and acceptor-labeled FtsL. Then, the stoichiometry of the complex was obtained by monitoring FRET between equimolar donor- and acceptor-labeled FtsB TM peptides as the relative concentration of an unlabeled FtsL-TM peptide was increased. The fact that direct FRET was observed between labeled FtsB molecules indicated that at least two FtsB monomers were present in the complex. In addition, the sharp saturation of the FRET efficiency observed once the amount of unlabeled FtsL added reached an equimolar FtsL:FtsB stoichiometry, indicating that the complex contains an equal number of FtsB and FtsL TM helices.

CONCLUSION

This review has emphasized the important role of FRET to study membrane protein interactions, discussing some of the achievements and the challenges associated with it. Over the last 30 years, FRET methods have greatly contributed to progress in understanding structure and interaction of membrane protein and to investigate their biological role. Various research groups have made key contributions to the growth of field by establishing methods and formalisms necessary to measure the interaction of TM helices in detergents and lipids and to compute free energies of association. This review has covered these contributions discussing the different combinations of fluorophores and membrane mimetics used, aiming to provide the reader with a comprehensive overview of the state of the art of this field.

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