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Förster resonance energy transfer in liposomes: Measurements of transmembrane helix dimerization in the native bilayer environment

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Abstract

The lipid bilayer vesicle is a model of the cellular membrane. Even in this simple system, however, measuring the thermodynamics of membrane protein association is a challenge. Here we discuss Förster resonance energy transfer (FRET) in liposomes as a method to probe the dimerization of transmembrane helices in a membrane environment. Although the measurements are labor intensive, FRET in liposomes can be measured accurately provided that attention is paid to sample homogeneity and sample equilibration. One must also take into account statistical expectations and the FRET that results from random colocalization of donors and acceptors in the bilayer. Without careful attention to these details, misleading results are easy to obtain in membrane FRET experiments. The results that we obtain in model systems are reproducible and depend solely on the concentration of the protein in the bilayer (i.e., on the protein-to-lipid ratio), thereby yielding thermodynamic parameters that are directly relevant to processes in biological membranes.

Free energy measurements of transmembrane (TM)[1] helix dimerization are needed for understanding the physical principles underlying vital cellular processes such as membrane protein folding and signal transduction. The folding of multispanning TM helices into unique three-dimensional structures [1–3] allows membrane proteins to carry out complex biochemical tasks. Lateral dimerization of receptor tyrosine kinases (RTKs) is a means of signal transduction across the plasma membrane and, therefore, is a key regulator of cell growth and differentiation [4–6].

During the past decade, new methods have been developed for thermodynamic studies of glycophorin A (GpA) [1,7] and have been applied to other TM dimers [4,8–14]. Russ and Engelman [15] developed a genetic assay termed TOXCAT for measuring self-association between TM helices in a biological context. The TOXCAT assay couples TM segment association with the expression of chloramphenicol acetyltransferase, which can be measured semiquantitatively. Fleming and co-workers [7] outlined the basis for studying the energetics of dimerization in detergent micelles by means of analytical ultracentrifugation. Fisher and co-workers [16] used Förster resonance energy transfer (FRET) to

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[1] *Abbreviations used:* TM, transmembrane; RTK, receptor tyrosine kinase; GpA, glycophorin A; FRET, Förster resonance energy transfer; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; FGFR3, fibroblast growth factor receptor 3; CD, circular dichroism; HFIP, hexafluoroisopropanol; TFE, trifluoroethanol; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; OCD, oriented circular dichroism; DOPC, dioleoylphosphatidylcholine; FRAP, fluorescence recovery after photobleaching; CMC, critical micelle concentration; SDS, sodium dodecyl sulfate.

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measure the association constant of GpA in detergents. FRET has also been used to assess the association of model TM helices in detergents [8,12].

FRET involves the nonradiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor [17–21] and is widely used as a spectroscopic tool for detecting molecular proximity. This method can be used to extract thermodynamic parameters of TM α-helix dimerization in detergent, as shown by Fisher and co-workers [16]. Here we use FRET as a tool for probing dimerization in liposomes, an environment that mimics the biological membrane.

We discuss sample preparation in detail and show that FRET can be measured reliably in liposomes. We demonstrate that the data are reproducible and relevant to processes occurring in biological membranes. We discuss statistical limitations to the magnitude of the observed FRET, and we also show how to interpret the data. The latter is a challenge due to the inevitable FRET that arises from random colocalization of donors and acceptors in vesicles.

Materials and methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The TM domain used here as a model is the fibroblast growth factor receptor 3 (FGFR3) TM domain, which has the amino acid sequence DEA GSVYAGILSYGVGFFLFILVVAAVTLCRLR. The peptide was custom synthesized at the Kansas State Biotechnology Facility and was purified using reverse-phase HPLC and water/acetonitrile gradient as described previously [22]. The peptides were labeled with two different donor/acceptor dyes: fluorescein/rhodamine and Cy3/Cy5. The dyes were attached to Cys396, a naturally occurring Cys in the TM domain of FGFR3.

Circular dichroism

Circular dichroism (CD) spectra of the peptide were collected using a Jasco 710 spectropolarimeter. The concentrations of the peptides in the samples, required for calculating molar ellipticities, were determined from absorbance measurements in a Cary 50 (Varian) spectrophotometer.

Preparation of vesicles

Lipids and proteins were first mixed in organic solvents (hexafluoroisopropanol (HFIP)/trifluoroethanol (TFE)/chloroform). Solvents were removed under a stream of nitrogen gas, and the mixture was lyophilized and then redissolved in 10 mM sodium phosphate buffer, 500 mM NaCl, pH 7. FRET was measured in three different liposomal systems:

Multilamellar vesicles

The hydrated lipid was freeze–thawed several times, and FRET was measured without further manipulation of the multilamellar vesicles (MLVs).

Extruded large unilamellar vesicles

The liposomal samples were extruded using a 100-nm pore diameter membrane (Avanti) to produce large unilamellar vesicles (LUVs). The final concentration of the proteins in the LUV solutions was determined from absorbance measurements using a Cary UV/Vis spectrophotometer (Varian). The lipid concentration was determined using a standard phosphate assay.

Small unilamellar vesicles

MLVs were sonicated in a bath sonicator to produce small unilamellar vesicles (SUVs).

Förster resonance energy transfer

FRET experiments in vesicles were carried out using a Fluorolog fluorometer (Jobin Yvon). For the fluorescein/rhodamine donor/acceptor pair, the excitation wavelength was set at 439 nm and emission spectra were collected from 450 to 800 nm (Fig. 1A). For Cy3/Cy5 samples, the excitation wavelength was set at 500 nm and emission spectra were collected from 540 to 800 nm (Fig. 1B). FRET was measured in liposomes containing known concentrations of donor- and acceptor-labeled proteins. Liposomes containing only donor-labeled proteins served as the "no FRET" control. Energy transfer, E, was calculated from measurements of donor intensity at 568 nm (for Cy3/Cy5) or 519 nm (for fluorescein/rhodamine) in the absence and presence of the acceptor according to

$$E(\%) = (I_{\mathrm{D}} - I_{\mathrm{DA}})/(I_{\mathrm{D}}) \times 100, \quad (1)$$

where I_{D} and I_{DA} are the donor intensities of samples containing only donor-labeled proteins and samples with both donor- and acceptor-labeled proteins, respectively.

Results

Labor intensiveness of FRET measurements in liposomes

In detergent samples, peptide and detergent molecules exchange between micelles very quickly. Therefore, to measure FRET, one can start with a sample of donor-labeled peptide in detergent and titrate a detergent solution of acceptor-labeled peptide. With highly

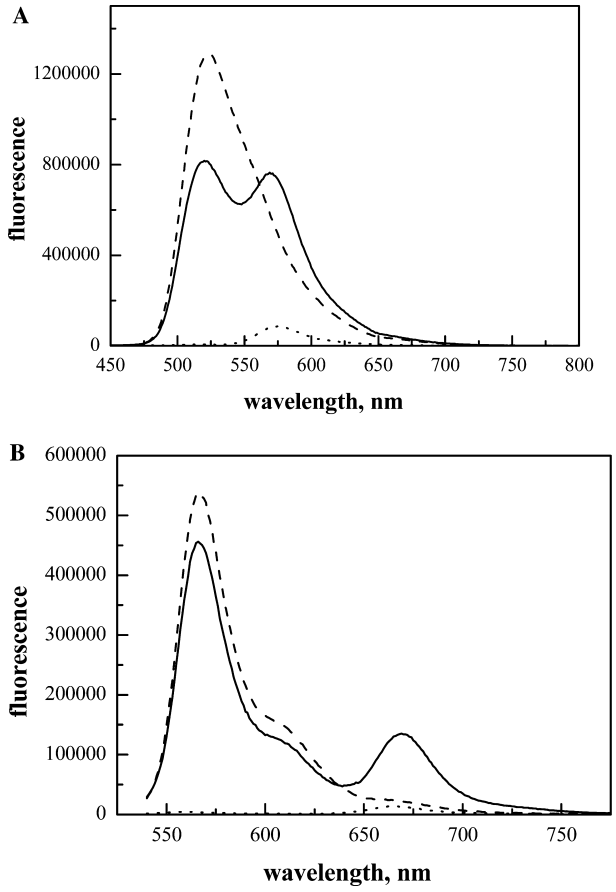

Fig. 1. Fluorescence spectra of donor- and acceptor-labeled peptide mixtures in liposomes. Spectra were measured for samples containing donor- and acceptor-labeled peptides (solid lines) as well as control samples containing only donor-labeled peptides (dashed lines) and only acceptor-labeled peptides (dotted lines). (A) A characteristic spectrum for the fluorescein/rhodamine pair. The excitation was fixed at 439 nm, such that only fluorescein was directly excited. The emission was scanned from 450 to 800 nm. FRET is obvious (solid lines) from the decrease in fluorescein fluorescence (~520 nm) and the appearance of sensitized rhodamine fluorescence (~570 nm). Percentage FRET was calculated from the decrease in fluorescein fluorescence at 519 nm (Eq. (1)). (B) A characteristic spectrum for the Cy3/Cy5 pair. The excitation was fixed at 500 nm, such that only Cy3 was directly excited. The emission was scanned from 540 to 800 nm. FRET (solid lines) results in a decrease of Cy3 fluorescence (~570 nm) and the appearance of sensitized Cy5 fluorescence (~670 nm). FRET efficiencies were calculated from the decrease in Cy3 fluorescence at 568 nm (Eq. (1)).

hydrophobic peptides in liposomes, however, we cannot do titration experiments. Titrating liposomes with acceptor-labeled peptides into liposomes with donor-labeled peptides will not lead to their equilibration. Instead, each sample must be prepared separately from stocks of lipids and donor- and acceptor-labeled proteins. A new sample must be prepared for each parameter such as peptide-to-lipid ratio, donor-to-acceptor ratio, or peptide concentration. Furthermore, for each measurement, we need three different samples: (i) a sample containing both donor and acceptor, (ii) a "no FRET" control containing the donor only, and (iii) an acceptor-only control to monitor the direct excitation of the acceptor fluorophore. Therefore, FRET measurements in liposomes are much more tedious than FRET measurements in detergents.

Confirming TM orientation of the peptides in the bilayer

In detergent micelles, the detergent molecules are expected to pack around the TM domains into a spherical or ellipsoidal aggregate. Therefore, we usually do not worry about the orientation of the TM helix in the spherical micelle. In liposomes, however, one must prove that the orientation of the helix is indeed TM. Tilt of the helix with respect to the bilayer normally can be easily measured using oriented circular dichroism (OCD) in oriented multilayers prior to hydration. As shown previously, the OCD spectra for helices that are normal and parallel to the bilayer plane are dramatically different [23–25]. Helices that are parallel to the membrane plane exhibit two minima at 205 and 225 nm and a maximum around 192 nm. TM helices, however, exhibit a single minimum around 230 nm and a maximum around 200 nm.

If the organic solvent used does not dissolve the two components (lipids and peptides) equally well, the helices may get "trapped' at the interface, perhaps in the form of peptide aggregates, and the OCD spectrum will reveal a mixture of TM and interfacial conformations. We have observed, however, that a mixture of HFIP/TFE/chloroform is a good solvent for both TM peptides and lipids and ensures the TM orientation of the peptides [22].

Sample homogeneity

When peptides and lipids are mixed in organic solvents, they can either (i) form a homogeneous mixture (i.e., a single "phase") or (ii) segregate into two or more distinct lipid- and peptide-rich phases. The FRET method relies heavily on the assumption that the two components can be completely and thoroughly mixed to form a single phase. Therefore, homogeneity of peptide/lipid mixtures should be assessed using different methods such as X-ray diffraction, fluorescence microscopy, and FRET efficiencies as described below.

Phase separation in lipid systems is easily "diagnosed" with X-ray diffraction. A homogeneous sample gives rise to a single set of Bragg peaks. A phase-separated sample shows either (i) two sets of Bragg peaks or (ii) a single set of Bragg peaks, identical to pure lipid samples, and one or several sharp lines due to protein aggregates. Our previous work (unpublished results) has demonstrated that phase separation is particularly likely to occur in dry samples. Therefore, X-ray diffraction of dry samples provides a very stringent test for possible phase separation.

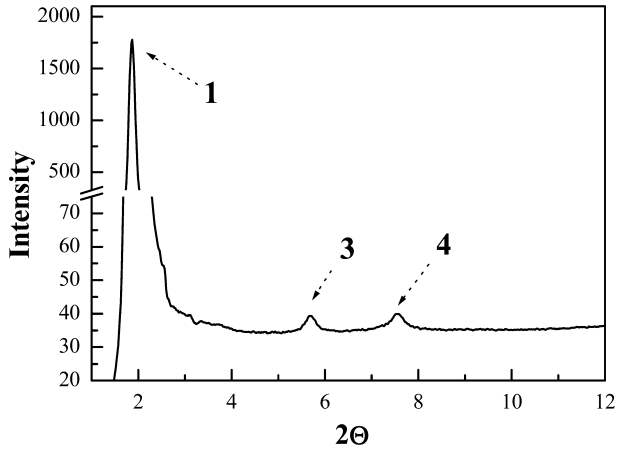

Fig. 2. Raw X-ray data for dry DOPC bilayers containing 5 mol% peptide, showing a single set of Bragg peaks. No phase separation between lipids and peptides was observed using X-ray scattering. The arrows point to the positions of the first, third, and fourth diffraction orders. The intensity of the second order is zero.

Fig. 2 shows raw X-ray data for dry dioleoylphosphatidylcholine (DOPC) bilayers containing 5 mol% peptide. The "raw" intensity versus 2Θ plots show a single set of Bragg peaks. The relative intensities of the peaks differ substantially from intensities recorded for dry DOPC, characterized by a very large fourth peak (data not shown). Therefore, proteins and lipids appear to be thoroughly mixed and form a single phase.

Next we investigated whether macroscopic phase separation occurs in single bilayers containing the TM domains using fluorescence microscopy. We prepared unilamellar POPC liposomes containing Cy3-labeled TM domains and NBD–PE. Vesicles were incubated with clean microscope glass slides for 30 min, and the excess vesicles were removed via extensive rinsing with buffer, leaving a bilayer-coated glass surface. NBD–PE and TM–Cy3 were imaged using the appropriate filters [26]. To prove that the imaged structures were single bilayers, we measured lipid diffusion coefficients using fluorescence recovery after photobleaching (FRAP). The fluorescence images were analyzed with the public domain software ImageJ, and the calculated diffusion coefficients were approximately 2×10^{-8} cm^2/s, exactly the values expected for lipid bilayers. The fluorescence images of both Cy3 and NBD appeared to be homogeneous at the micron scale [26].

Aggregation of the proteins due to their dissolution from the lipid matrix can be further detected by measuring FRET as a function of acceptor concentration. It has been shown that if the helices form dimers but no higher order aggregates (such that only monomers and dimers are present), FRET depends linearly on the acceptor ratio [27,28] but a larger aggregate either in or out of the bilayer will have a nonlinear dependence on acceptor concentration. In our model system, FRET depends linearly on acceptor concentration [22] (data not shown).

Multilamellar vesicles

After hydration, the multilamellar liposome solutions were subjected to freeze–thaw cycles, a method routinely used to achieve equilibration. We observed that after one cycle, the turbidity of the samples was obviously reduced. Such a substantial decrease in turbidity is surprising given that it does not occur for lipid MLVs that do not contain the protein. Therefore, it appears that the presence of the peptide is promoting the formation of relatively small MLVs. This is true for peptide concentrations ranging from 0.01 to 1 mol%. Fig. 3 shows the UV absorbance (A) and the CD spectrum (B) of an MLV sample containing 0.9 mol% Cy5-labeled peptide (labeling yield ~30%) that has been cycled three times

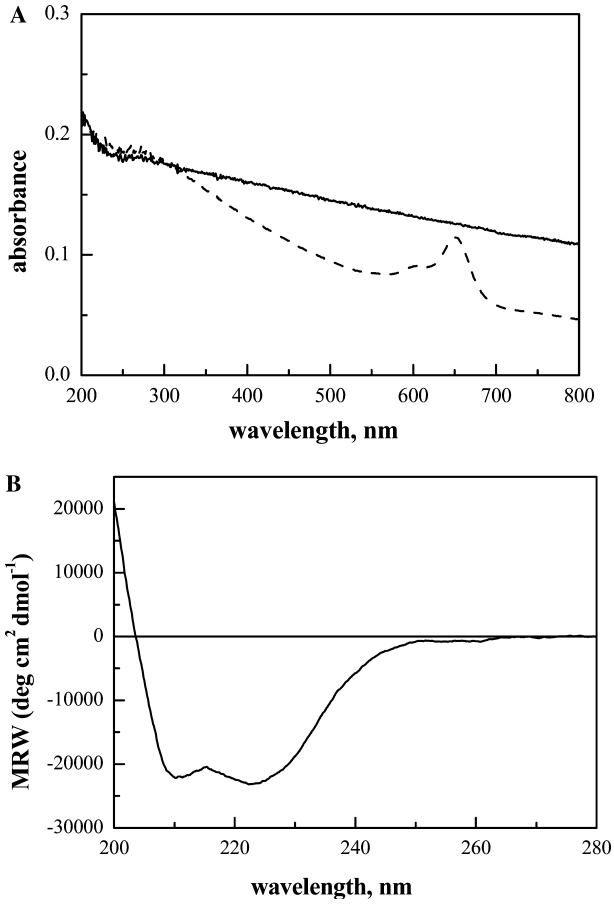

Fig. 3. Absorbance spectrum (A, dashed line) and CD spectrum (B) of MLVs containing 0.9% peptide (optical path = 0.1 cm). The peptide was labeled with Cy5, and the labeling yield was 27%. The MLVs were freeze–thawed three times, and the two spectra were recorded without further MLV manipulation. Also shown is the absorbance spectrum of a lipid-only MLV sample (solid line in A). Light scattering did not prevent us from recording a meaningful CD spectrum of the peptide in MLVs.

through the main phase transition. As shown in Fig. 3, the absorbance is relatively low, such that a CD spectrum with a high signal-to-noise ratio can be collected. Although the CD spectrum is likely affected by scattering for wavelengths less than 210 nm, the quality of the spectrum is good enough that a conclusion can be drawn with confidence that the peptides are helical. The ability to draw such a conclusion is critical because TM helices may misfold and aggregate into β-sheets. Therefore, it is recommended that the CD spectrum be recorded for each sample. As discussed above, the TM orientation in the bilayer should be confirmed using OCD.

Having shown above that the TM peptides are homogeneously distributed when MLVs are prepared, we next assessed whether they remain homogeneous over time. We observed that the fluorescence intensity and the FRET signal of such MLVs are very stable. Fig. 4 shows the FRET signal for rhodamine- and fluorescein-labeled TM helices. The liposome samples were freeze–thawed once, and the FRET signal was measured (solid line). After two additional freeze–thaw cycles, the fluorescence spectrum was measured again (open circles). The two spectra are identical, suggesting that the samples are fully equilibrated after only one freeze–thaw cycle. To further assess signal stability, samples were kept in the refrigerator for 1 month, after which FRET was measured and was found not to have changed.

To determine whether MLVs can be used for FRET measurements, we investigated the effect of light scattering from MLVs on the fluorescence spectra such as the spectrum shown in Fig. 1. Fig. 5 compares the observed intensity for two samples containing 0.3 mol% protein. One of the samples (dashed line) had unlabeled peptides and served as a "scattering control," whereas the second sample (solid line) contained rhodamine- and fluorescein-labeled peptides (1:1 ratio). Comparison of the two spectra suggests that the measured fluorescence intensity does not have a sizable contribution due to scattering. In addition, we showed that scattering does not reduce the fluorescence intensity. Fig. 6 shows the spectrum of an

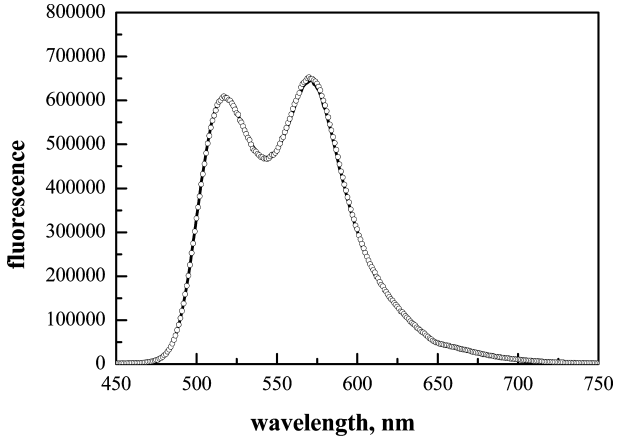

Fig. 4. FRET spectra recorded for 0.15 mol% fluorescein-labeled peptide and 0.15 mol% rhodamine-labeled peptide in MLVs. The MLVs were freeze–thawed once, and the FRET signal was measured (solid line). After two additional freeze–thaw cycles, the fluorescence spectrum was measured again (open circles). The two spectra are identical, suggesting complete equilibration after hydration and a single freeze–thaw cycle.

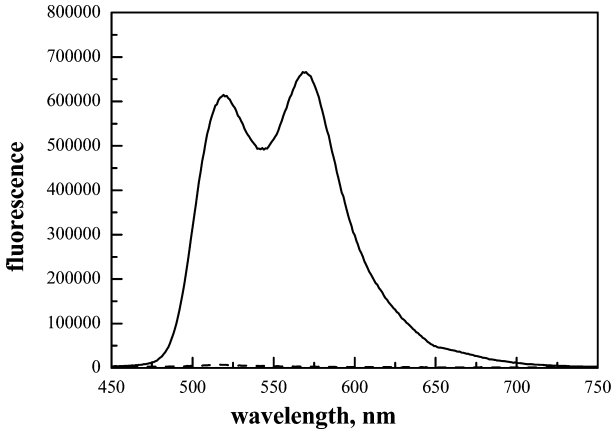

Fig. 5. Contribution of scattering to the measured FRET spectra. Solid line: MLVs containing 0.1 mol% fluorescein-labeled peptide and 0.1 mol% rhodamine-labeled peptide. Dashed line: MLVs containing 0.2 mol% unlabeled peptide. The latter sample serves as a control to quantify the effect of scattering on the recorded FRET spectra. Comparison of the two spectra suggests that the contribution from scattering does not exceed a fraction of a percentage point and therefore is negligible.

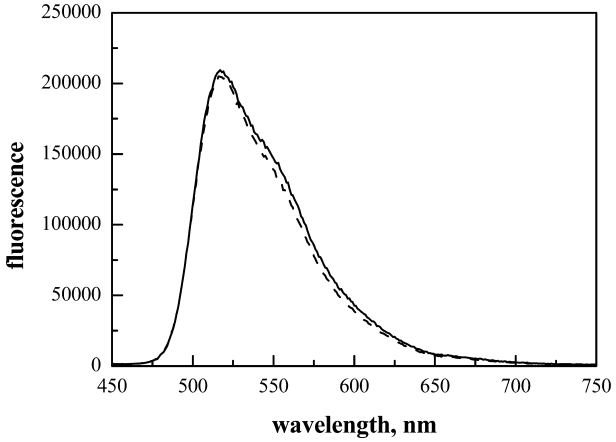

Fig. 6. Fluorescence intensity of fluorescein-labeled peptides in MLVs before (solid line) and after (dashed line) the addition of Triton X-100. Lipid concentration was 1 mg/ml, and the sample contained 0.04 mol% fluorescein-labeled peptide. Triton X-100 was added to the sample to solubilize the MLVs and reduce the turbidity. Typically, the absorbance of the samples at 525 nm decreases from 0.9 to 0.3 (optical path = 1 cm) on the addition of Triton X-100. The addition of Triton X-100 did not cause a statistically significant change in fluorescence amplitude, suggesting that scattering does not reduce the fluorescence intensity. If scattering were affecting the measurements, the fluorescence amplitude would have increased after the addition of Triton X-100.

MLV sample containing fluorescein-labeled peptides (solid line). To eliminate light scattering from vesicles, Triton X-100 was added to the sample to solubilize the MLVs (decreasing the absorbance from ~0.9 to ~0.3 at 525 nm for optical path 1 cm), and the spectrum was remeasured (dashed line). The change in fluorescence intensity after the addition of Triton X-100 was insignificant. Therefore, we conclude that scattering does not affect the fluorescence intensity in our experimental system, so that MLVs can be used to measure FRET in vesicles.

Finally, we demonstrated that the FRET efficiency in MLVs is determined only by the protein-to-lipid ratio, not by the total peptide and lipid concentrations (see Fig. 8A). Results are discussed in detail below.

Large unilamellar vesicles

The LUV is a system that is widely used as a model for measurements of protein binding to membranes and insertion into membranes [29,30]. Therefore, we sought to assess the relevance of this system for FRET measurements of TM helix dimerization. To prepare LUVs, we extruded the MLV samples through a 100-nm pore membrane. To calculate FRET in liposomes, both donor-only and donor–acceptor samples were extruded and FRET was calculated according to Eq. (1). As shown in Fig. 7, we observed no statistically significant decrease in FRET in LUV compared with MLV.

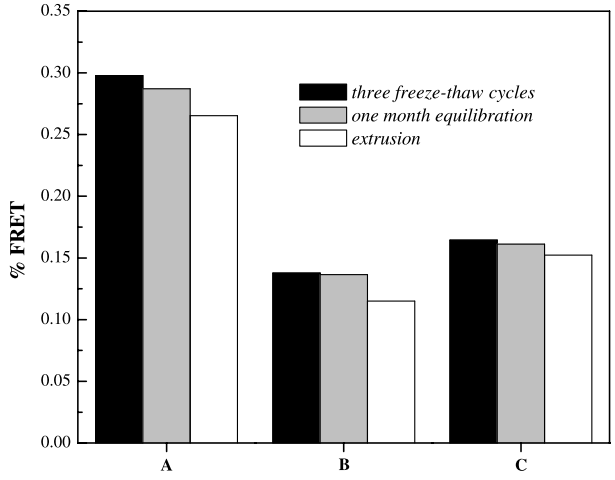

Fig. 7. Measured FRET efficiencies after a single freeze–thaw cycle (black), after 1 month equilibration (gray), and subsequent extrusion to produce LUVs (white) for three samples. Sample A: 0.1 mol% fluorescein-labeled peptide and 0.1 mol% rhodamine-labeled peptide. Sample B: 0.05 mol% fluorescein-labeled peptide and 0.05 mol% rhodamine-labeled peptide. Sample C: 0.04 mol% fluorescein-labeled peptide and 0.04 mol% rhodamine-labeled peptide. Lipid concentration was 1 mg/ml for all samples. After 1 month, we observed no change in the measured FRET efficiency. After extrusion, percentage FRET decreased from 28.7 to 26.5% for sample A, from 13.6 to 11.5% for sample B, and from 16.1 to 15.2% for sample C. We consider this decrease negligible.

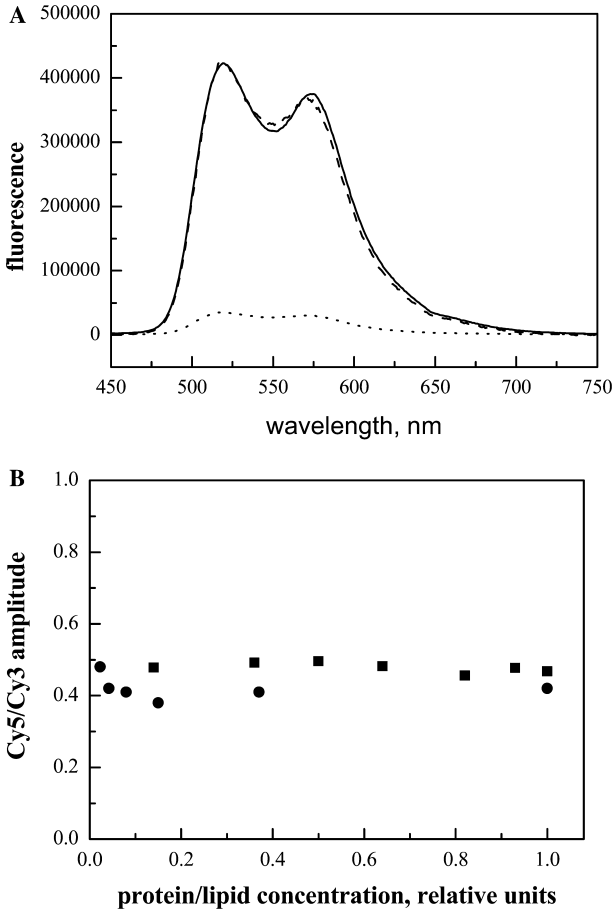

Fig. 8. (A) FRET signal for two MLV samples with the same peptide-to-lipid ratio but different buffer volumes. A common lipid-to-protein stock was prepared to ensure an identical peptide-to-lipid ratio. The stock was divided into two samples of volumes 50 and 600 μl, the organic solvent was evaporated, and the two samples were hydrated in 400 μl sodium phosphate buffer, such that one of the samples was 12 times more diluted than the other. Both samples contained 0.1 mol% fluorescein-labeled peptide and 0.1 mol% rhodamine-labeled peptide per mole of lipid. Solid line: FRET spectrum of the "concentrated" sample. Dotted line: FRET spectrum of the "diluted" sample. Dashed line: FRET spectrum of the diluted sample is multiplied by 12 and is identical to FRET spectrum of the concentrated sample. The results suggest that the FRET efficiency is determined by the protein-to-lipid ratio but not by the total peptide and lipid concentrations. (B) Ratio of Cy5 (acceptor) to Cy3 (donor) amplitude for two sets of extruded LUV samples for various peptide/lipid concentrations. In each set, the peptide-to-lipid ratio is the same, whereas the total peptide/lipid concentration is different. The most concentrated sample is assigned a relative concentration of 1, and the concentrations of the diluted samples are scaled accordingly. Set 1, squares: 0.4 mol% peptide, Cy3-to-Cy5 ratio = 1; Set 2, circles: 0.3 mol% peptide, Cy3-to-Cy5 ratio = 1. The Cy5/Cy3 amplitude does not change for dilutions up to 32 times, demonstrating that the peptide-to-lipid ratio is the sole determinant of FRET efficiency in LUVs.

Therefore, FRET efficiencies and helix–helix interactions measured in MLVs and LUVs are comparable.

A potential problem with extrusion is the loss of proteins and lipids in the extrusion process. This is why protein and lipid concentrations are generally different from

Table 1
Loss of peptides and lipids during extrusion

	Sample A	Sample B	Sample C
Peptide loss (%)	17	13	18
Lipid loss (%)	18	14	15
R_{after}/R_{before}	1.01	1.01	0.99

Peptide loss was determined from measurements of absorbance and fluorescence spectra before and after extrusion. Lipid concentration before and after extrusion was determined using a standard phosphate assay. R_{before} and R_{after} are the peptide-to-lipid ratios before and after extrusion. A value of $R_{after}/R_{before} = 1$ indicates that the peptide-to-lipid ratio does not change due to extrusion. Samples A and B are two different samples containing 0.1 mol% peptide. Sample C contains 0.2 mol% peptide.

the intended ones and must be measured after extrusion. We determined protein concentration in the LUVs by measuring dye absorbance or fluorescence and the lipid concentration using a standard phosphate assay (Table 1). We found that typical losses of proteins and lipids were identical; that is, between 13 and 18% of both lipids and proteins were lost during the extrusion process. Because the percentage losses were similar, the protein-to-lipid ratio remained the same (Table 1). Despite material loss, one can still do quantitatively meaningful experiments if only the protein-to-lipid ratio, but not the water content, is the determinant of the FRET efficiency. Therefore, we expanded the MLV study in Fig. 8A to extruded vesicles. We started with a common lipid/protein solution in organic solvent (Cy3-to-Cy5 ratio of 1). Then we added different amounts of buffer to identical dried aliquots of the lipid/peptide stock, and we collected fluorescence spectra for various buffer dilutions. In Fig. 8B, we plot the ratio of the acceptor-to-donor peaks (Cy5 to Cy3) as a measure of FRET for two different samples (different protein-to-lipid ratios). The Cy5/Cy3 amplitude does not change for dilutions up to 32 times. Therefore, FRET does not depend on the buffer volume; rather, it depends only on the protein-to-lipid ratio.

The observation that the FRET efficiencies depend solely on the protein-to-lipid ratios has an important implication. It suggests that we can directly probe the monomer/dimer protein equilibrium in the bilayer. In liposomes, the protein acts as a solute and the lipid matrix acts as a solvent, whereas water is a separate phase that has no effect on the effective concentration of peptide in the membrane. Therefore, FRET measurements in liposomes allow us to extract "absolute" thermodynamic parameters pertaining to dimerization of TM helices in lipid membranes.

Small unilamellar vesicles

SUVs were produced via sonication of MLVs. When we attempted to measure FRET in SUVs, we noticed a decrease in the amplitude of the fluorescence spectra.

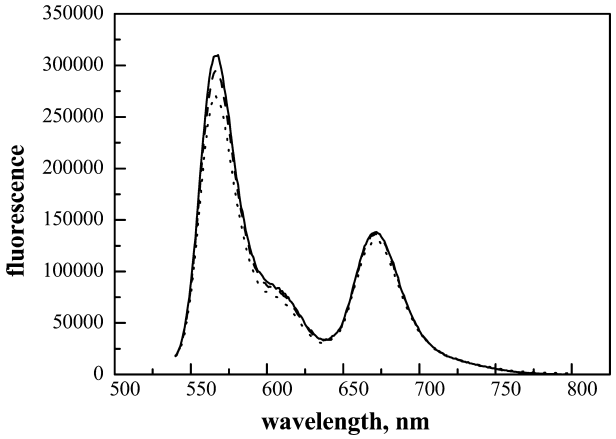

Fig. 9. Fluorescence spectra of Cy3- and Cy5-labeled peptide mixtures in SUVs. Two of the spectra (dashed line and dotted line) were acquired 5 and 10 min, respectively, after the first spectrum (solid line). The amplitude of the signal decreased steadily over time, suggesting that SUVs are not an appropriate system for FRET measurements.

This result is shown in Fig. 9, where two of the spectra, shown with the dashed and dotted lines, were acquired in 5 and 10 min, respectively, after the first spectrum (solid line). In this experiment, the cuvette was not moved and the sample was not manipulated in any way between spectra acquisitions. We note that such a decrease in signal amplitude, characteristic of SUVs, is not observed for MLVs or LUVs. Judging by the shape of the spectra (i.e., by the ratio of Cy3-to-Cy5 fluorescence), the FRET efficiency does not change as the amplitude decreases. SUVs are known to be far from equilibrium, and we believe that the SUVs are aggregating and coming out of solution. Although the exact mechanism is unknown, our results suggest that SUVs are not an appropriate system for FRET measurements, particularly if Eq. (1) is used to calculate FRET efficiencies.

Discussion

Summary of findings

The energetics of TM helix dimerization has been studied primarily in detergent using analytical ultracentrifugation [7,31] or FRET [16]. The detergent environment offers an important advantage, namely that detergent molecules and proteins equilibrate rapidly. The latter is a requirement for the analytical ultracentrifugation technique. Furthermore, FRET experiments in detergent are relatively easy to carry out by titrating the acceptor into a donor-containing suspension and measuring FRET after each addition of acceptor.

Here we have shown that FRET can be measured in lipid vesicles. MLVs, prepared by premixing the proteins and lipids in organic solvent and hydrating the mixture, are in equilibrium after a single freeze–thaw cycle (gen-

erally required for equilibration of buffers, salt, etc.). The FRET spectrum recorded after one freeze–thaw cycle is the same as after two or more additional freeze–thaw cycles or after a month-long equilibration. The MLVs can be further extruded to produce LUVs. Typically, we observed a small loss of material with no change in peptide-to-lipid ratios and a slight (2–5%) decrease in measured FRET efficiencies after extrusion. This slight decrease, on the order of the typical experimental error, may be explained by eliminating the possibility of FRET occurring between donor and acceptor in different bilayers in the multilamellar samples.

The fact that the measured FRET efficiency depends solely on the peptide-to-lipid ratio is a major advantage of FRET measurements in liposomes. This result suggests that the thermodynamic parameters derived from FRET measurements in liposomes are likely directly relevant to processes occurring in cellular membranes. In detergents, the measured free energies depend not only on the protein-to-detergent ratio but also on the total detergent concentration and the critical micelle concentration (CMC) [32]. For detergents, the CMC is dependent on many experimental details, including the nature of the TM helix, and cannot be known or measured accurately in most experimental systems. This is especially true for ionic detergents such as sodium dodecyl sulfate (SDS). Because TM helices associate with micelles and not with monomeric detergent, the unavoidable uncertainty in detergent CMC gives rise to an uncertainty in the effective peptide concentration. The CMC of a phospholipid is extremely low [33] and thus is negligible. The peptide-to-lipid ratio in a bilayer experiment is equal to the system composition.

FRET for parallel and antiparallel dimers

The efficiency of energy transfer E is inversely proportional to the sixth power of the distance, r, between donor and acceptor. The transfer efficiency E is a function of r and R_0, the characteristic Förster radius for the donor/acceptor pair, and is given by

$$E = \frac{1}{1 + (R_0/r_i)^6}. \quad (2)$$

The donor/acceptor pairs used in this work, Cy3/Cy5 and fluorescein/rhodamine, have R_0 of 55 Å. For this value of R_0 and $r = 10$ to 20 Å (typical for a helix parallel dimer, helix diameter is 10 Å), E is 99%. In an antiparallel dimer, the transfer efficiency E will depend on the distance between donor and acceptor. If the dyes are attached to the N termini, they may be as far as 50 Å apart; in this case, E would be 70%. In any case, E will be no less than approximately 70% for antiparallel dimers of TM helices. In the biological systems that we are studying, the homodimers are usually parallel dimmers; therefore, we consider only parallel dimers in our discussion.

Self-quenching of donors

Self-quenching of donors could be a problem for dyes with overlapping emission and excitation spectra such as fluorescein (but not Cy3). For instance, a value $R_0 = 44$ Å has been reported for fluorescein/fluorescein self-quenching (Molecular Probes, www.probes.com). To investigate whether self-quenching contributes to the observed decrease in donor fluorescence, we measured the fluorescence intensity of MLVs containing various concentrations of fluorescein-labeled peptides (Fig. 10). The amplitude is linear with peptide concentration, suggesting that the self-quenching effects are negligible compared with FRET. We also measured the fluorescence amplitude in these MLVs in the presence and absence of unlabeled peptides. The presence of unlabeled peptides has no effect on the fluorescence spectra of both (i) fluorescein-labeled TM_{FGFR3} (data not shown) and (ii) a sequence variant of TM_{FGFR3} with a much stronger dimerization propensity (unpublished observations). Based on these experiments, we conclude that fluorescein self-quenching does not affect the measured FRET efficiency. Such control experiments should always be carried out for donor dyes with overlapping emission or excitation spectra.

FRET due to random acceptor/donor colocalization

When measuring FRET in a fluid lipid bilayer, it is important to recognize that FRET can arise simply due to random proximity of the acceptors and donors. Therefore, it is desirable to use low peptide concentration, such that the average distances between the pep-

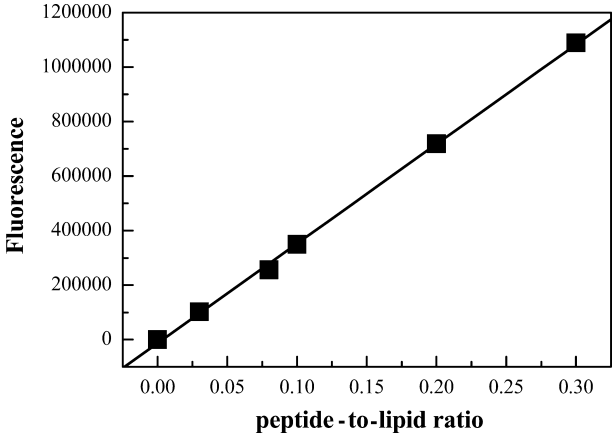

Fig. 10. Fluorescence amplitude (at 521 nm) of MLVs as a function of fluorescein-labeled TM_{FGFR3} concentration. The amplitude increases linearly with the amount of fluorescein in the sample, suggesting that self-quenching of fluorescein does not contribute to a measurable decrease in fluorescein fluorescence under the conditions used in the FRET experiments. This finding is further supported by the lack of changes in fluorescence intensity after the addition of unlabeled peptides (see text).

tides always exceed the Förster radii for the donor/acceptor pair. It should be further taken into account that the peptides diffuse randomly in the bilayers, such that for any concentration some acceptors will come in close contact with donors and FRET will occur. Therefore, one must perform control experiments to determine whether the measured FRET signal is at least in part due to dimerization.

An indicator of what proportion of the measured FRET efficiency is due to dimerization rather than random colocalization is the deviation of the measured FRET from expected FRET calculated for randomly distributed fluorophores. In this calculation, carried out first by Wolber and Hudson [34] and simulated by Wimley and White [35] and by Li and co-workers [22], FRET from randomly distributed peptides is determined by averaging the donor quenching by acceptors in a specific configuration over a large number of acceptor configurations. The efficiency of FRET (E) of a donor by a specific acceptor configuration is given by

$$E_{\text{colocalization}} = \frac{1}{1 + \sum_i (R_0/r_i)^6}, \tag{3}$$

where r_i is the distance between the donor and the ith acceptor in the system and R_0 is the Förster radius for the donor/acceptor pair. The simulation of FRET from random colocalization as the average of a large number of random acceptor configurations is shown in Fig. 11 for various R_0 values and acceptor concentrations. Importantly, these data show that this random proximity effect in bilayers can give a significant amount of FRET even at acceptor concentrations far less than 1 mol% in the bilayer. This nonspecific FRET is maximal when fluorophores with large R_0 values are used. Furthermore, the quenching of the donor fluorescence by acceptors on the opposite bilayer leaflet increases E due to colocalization by an additional 10% or so for $R_0 = 55$ Å, whereas little effect is expected for smaller R_0 values of 25 to 30 Å. Therefore, the contribution from transbilayer FRET is relatively small.

In typical bilayer FRET experiments, including those presented here, the measured FRET will always contain contributions from dimerization *and* from random colocalization. To obtain the FRET efficiency that is due to dimerization, one must subtract FRET due to proximity from the measured FRET signal. This is illustrated in Fig. 12, which shows the measured FRET (solid squares, data from [22]) and the corrected FRET that reports dimerization (open circles). For the correction, we have used $R_0 = 55$ Å (Molecular Probes). We note that the corrected data still must be divided by $E^{\text{dimer}}_{\text{expected}}$, according to Eqs. (4) and (5) (discussed below).

A control experiment that also helps to distinguish between random colocalization and true dimerization is to monitor the effect of "dilution" of labeled peptides with unlabeled peptides. If sequence-specific dimerization occurs, the addition of unlabeled peptide to donor- and acceptor-labeled dimers will decrease the FRET signal (see Eq. (4) below). FRET that is due to random colocalization will not decrease in the presence of unlabeled peptide. This control experiment has demonstrated that GpA forms sequence-specific dimers in SDS [16].

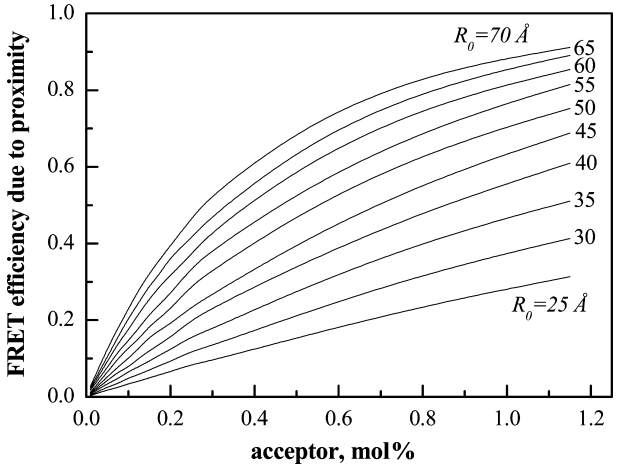

Fig. 11. Numerical simulation of nonspecific FRET in the membrane. FRET from randomly distributed peptides was calculated by averaging the donor quenching by acceptors in a specific configuration over 1000 different random acceptor configurations. Each line is the FRET expected from random colocalization of acceptors and donors in membranes for a specific R_0 value. FRET was calculated at different concentrations of acceptors in the bilayer up to 1 mol%. Notice the substantial amount of FRET for large R_0 values, even at very low concentrations.

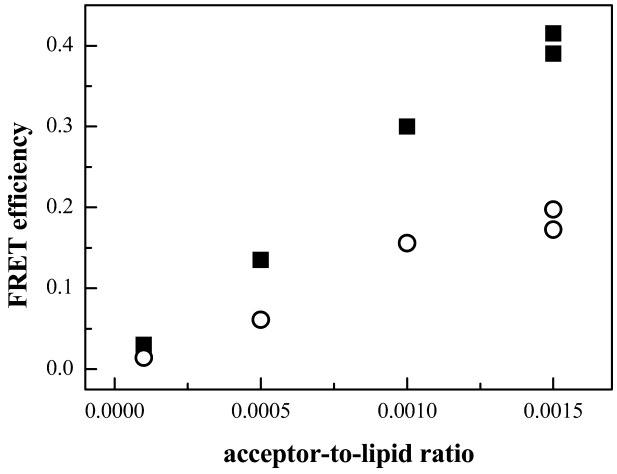

Fig. 12. For a quantitative description of dimerization, the measured FRET efficiency (solid squares, data from [22]) needs to be corrected for random colocalization of donors and acceptors according to Eq. (3). The corrected data (open circles) report sequence-specific dimerization. These data are for an equimolar mixture of fluorescein- and rhodamine-labeled peptides in bilayers. A value of $R_0 = 55$ Å was used for the correction. The maximum possible FRET for this system is 50%, and the fraction dimer reaches approximately 40% at the highest peptide concentrations studied.

Statistical expectations

Making thermodynamic measurements of dimerization requires a quantitative statistical understanding of the maximum amount of FRET that one can expect to observe for a particular sample. Given the simplest case of homodimerization of TM helices that are labeled with either acceptor or donor, there are statistical and experimental limitations on the amount of FRET that can be observed. For a TM helix length of approximately 30 Å and typical FRET R_0 values greater than 50 Å, we can assume that each dimer that contains an acceptor and a donor has 100% FRET efficiency. Nonetheless, two sources decrease the maximum possible FRET from 100%. First, there are nonproductive donor/donor pairs. In a random equimolar mixture, these are as likely to form as are FRET-producing acceptor/donor pairs. Second, one must take into account the presence of unlabeled peptide molecules. It has been our observation that the efficiency of labeling TM helices with some fluorescent dyes is often poor, sometimes as low as 30%. Unless care is taken to measure the dye loading of a peptide, it is easy to conclude erroneously that a brightly colored peptide sample is fully labeled. Although it is sometimes possible to get highly labeled peptides with aggressive and expensive labeling protocols, the yield of labeled peptides relative to total peptide is generally not 100%. The difficulty of purifying highly hydrophobic sequences such as TM helices further complicates the production of completely labeled peptides. Labeling efficiencies are often not reported in the literature, but it is likely that most FRET experiments on dimerizing helices are done at labeling efficiencies significantly less than 100%. For the fluorescein- and rhodamine-labeled peptides in this study, we achieved labeling efficiencies of 80 and 100%, respectively, by coupling with a very large excess of dye, using extended coupling times, and carefully monitoring the extent of coupling.

If acceptor-labeled peptides at a total concentration of $[P_A^{total}]$ have a labeling efficiency of f_A and donor-labeled peptides at a total concentration of $[P_D^{total}]$ have a labeling efficiency of f_D, the concentration of unlabeled peptide will be $[P_U] = [P_A^{total}](1 - f_A) + [P_D^{total}](1 - f_D)$ and the concentrations of labeled peptides will be $[P_A] = [P_A^{total}](f_A)$ and $[P_D] = [P_D^{total}](f_D)$. In a homodimerizing FRET sample, the following pairs will exist: P_AP_A, P_AP_D, P_DP_D, P_AP_U, P_DP_U, and P_UP_U. The relative proportion of each in a completely dimerized system can be calculated from peptide concentrations only if the labeling efficiencies are known. The maximum FRET possible for complete dimerization depends on the fraction of donors that are expected to have an acceptor for a partner relative to the total number of donors in the sample

$$E_{expected}^{dimer} = \left(\frac{P_DP_A}{P_DP_A + P_DP_D + P_DP_U}\right). \quad (4)$$

Thus, under typical experimental conditions, such as a sample containing an equimolar mixture of acceptor and donor-labeled peptides with 70% labeling efficiency, the *maximum possible* FRET is only 35%. Complete labeling of peptides will increase the maximum FRET to 50% in an equimolar mixture. Importantly, unlike FRET from dimerization, the FRET that arises from random colocalization of acceptors and donors in vesicles (see above) does not change in the presence of unlabeled peptide. Therefore, we can distinguish between FRET from true dimerization and FRET from random colocalization by adding unlabeled peptide.

Putting it all together

We have shown that there are advantages to measuring the dimerization of TM helices in bilayer membranes, as compared with measurements in detergent solutions, but that certain important facts must be accounted for to make quantitative calculations of dimerization from measurements of FRET. These facts include the FRET that arises from random colocalization and the statistical limitations on the amount of FRET that is expected for complete dimerization. In a membrane FRET experiment, the fraction of peptides that are in dimeric form can be calculated as follows:

$$\text{Fraction dimer} = \frac{E_{observed}^{total} - E_{colocalization}}{E_{expected}^{dimer}}. \quad (5)$$

Expressed in terms of mole fraction concentrations, X, this is equivalent to

$$\text{Fraction dimer} = \frac{\text{Dimer}}{\text{Total}} \text{ or } \frac{2X_{dimer}}{2X_{dimer} + X_{monomer}}, \quad (6)$$

where the factor of 2 accounts for the fact that there are two peptides per dimer and only one per monomer. Finally, mole fraction concentrations can be used to calculate a partition coefficient by $K_X = \frac{[X_{Dimer}]}{[X_{monomer}]^2}$ which is an equilibrium constant from which one can calculate free energy by $\Delta G_x = -RT\ln(K_x)$. Using this method, the free energy of dimerization for FGFR3 TM domain was calculated as approximately −3 kcal/mol [22].

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