Chapter 2

Measurement of Transmembrane Peptide Interactions in Liposomes Using Förster Resonance Energy Transfer (FRET)

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Abstract

Present day understanding of the thermodynamic properties of integral membrane proteins (IMPs) lags behind that of water-soluble proteins due to difficulties in mimicking the physiological environment of the IMPs in order to obtain a reversible folded system. Despite such challenges faced in studying these systems, significant progress has been made in the study of the oligomerization of single span transmembrane helices. One of the primary methods available to characterize these systems is based on Förster resonance energy transfer (FRET). FRET is a widely used spectroscopic tool that provides proximity data that can be fitted to obtain the energetics of a system. Here we discuss various technical aspects related to the application of FRET to study transmembrane peptide oligomerization in liposomes. The analysis is based on FRET efficiency relative to the concentration of the peptides in the bilayer (peptide:lipid ratio). Some important parameters that will be discussed include labeling efficiency, sample homogeneity, and equilibration. Furthermore, data analysis has to be performed keeping in mind random colocalization of donors and acceptors in liposome vesicles.

Key words Integral membrane proteins, Transmembrane helix, Energetics, Thermodynamic equilibrium, Free energy of association, FRET in liposomes

1 Introduction

Free energy measurements of transmembrane (TM) helix association in single and multispan membrane proteins are important to understand the mechanisms behind vital cellular processes such as membrane protein folding and signal processing [1]. However, research in the field of integral membrane proteins has considerably lagged behind in comparison to that of soluble proteins [2]. This is due to the difficulty in obtaining experimental systems that match the nature of their complex native environment, the bilayer, and conditions in which reversible association/unfolding can be established. Moreover, since the unfolded state of membrane proteins retains considerable amount of helical component [1] (in

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opposition to soluble proteins where the unfolded state largely lacks a secondary structure), monitoring unfolding is a significant challenge.

To overcome these challenges, a strong focus has been applied toward a more approachable folding question—the oligomerization of single-pass TM domains—a question that retains the core process that is important for the folding of membrane proteins, the association of the transmembrane helices. Several biophysical tools have been developed to measure TM helix associations in a number of environments, from detergent micelles to lipid vesicles and even biological membranes. Analytical ultracentrifugation (AUC), Förster resonance energy transfer (FRET), and thiol-disulfide exchange [2–4] are biophysical methods that are suitable for measuring the association of TM peptides in artificial environments. Genetic methods based on the conditional expression of a reporter gene such as TOXCAT [5] and GALLEX [6] are useful for measuring homo- and hetero-association of TM helices in the natural inner membrane of *Escherichia coli*.

In this chapter we focus on FRET-based studies of TM association in artificial liposomes. FRET involves excitation of the ground electronic state of a donor molecule followed by non-radiative transfer of energy from the excited state of the donor to an appropriate acceptor. Since FRET occurs only when two suitable fluorophores (a donor and acceptor molecule) are located within ~10 nm of each other, it can be used as a measure of molecular proximity [1, 4, 7]. The two fluorophores must have a significant spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor, a sufficiently high quantum yield, and a favorable dipole–dipole orientation (Fig. 1). When the fluorophores come in close proximity, energy is transferred non-radiatively, resulting in quenching of donor fluorescence and increase (sensitization) in acceptor fluorescence.

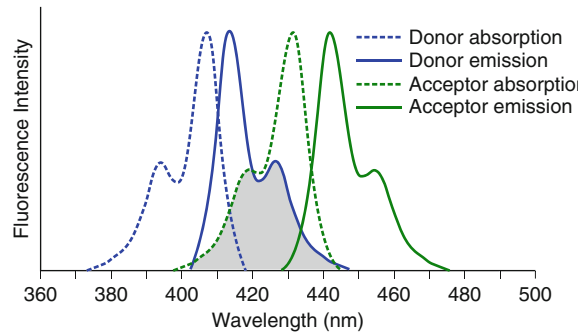

Fig. 1 Spectral overlap between a FRET pair. *Blue curves* depict the donor excitation and emission spectra and *green curves* depict the acceptor excitation and emission spectra. Note significant overlap (*shaded region*) between the donor emission and the acceptor excitation, leading to a good spectral overlap, one of the requirements for a FRET pair

In the case of TM helix–helix interactions, each peptide is labeled with either a donor or an acceptor molecule, and they are solubilized in artificial hydrophobic environments like detergents and lipids. Because TM peptides are insoluble in water and only experience the "hydrophobic volume" of the solution, their concentration is expressed as mole fraction, i.e., the number of moles of peptide relative to number of moles of the "true solvent" (detergent or lipid), yielding a peptide:detergent or peptide:lipid ratio. In addition to mole fractions, the second parameter that is often varied in a typical FRET experiment is the relative percentage of the donor- and acceptor-labeled peptides, which can help in determining the specific oligomeric state (i.e., dimer, trimer) of the system being studied [8]. Once a donor/acceptor system has been equilibrated, the FRET efficiency can be calculated by monitoring the degree of donor quenching in the presence of acceptor (as discussed in this chapter) or by monitoring the increase in acceptor emission. Spectral overlap between the donor and acceptor also causes contamination of the FRET signal due to Acceptor Spectral Bleedthrough, which refers to direct excitation of the acceptor by radiation at the donor excitation wavelength (Fig. 2). This must be subtracted from the acceptor emission intensity in the FRET samples while calculating FRET efficiency using acceptor sensitization (not used in this chapter).

FRET has been shown to yield association energetics in TM peptides in detergents [9] as well as lipids [3, 7]. It can also be used for studying hetero-oligomers in which cases the calculations are applied taking into account the various possible equilibria present in the system. This chapter discusses the experimental procedure to

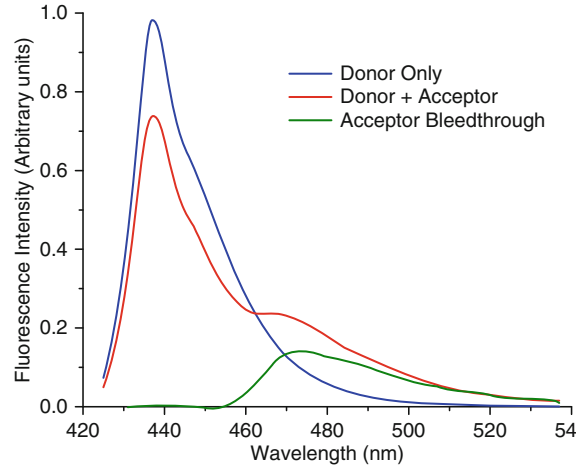

Fig. 2 Donor quenching and acceptor sensitization due to FRET. The decrease in the donor emission intensity is used to calculate the FRET efficiency. The sensitization of acceptor emission (FRET) is evident from the increased fluorescence intensity compared to direct excitation (acceptor bleedthrough) of the acceptor at the donor excitation wavelength

measure helix–helix *self* association in lipid vesicles and the relationship between mole fraction and FRET efficiency to calculate the association of affinity. An outline of the sample preparation techniques, including solid-phase peptide synthesis, labeling technique, HPLC purification, and MS characterization, is presented. This is followed by a detailed description of the FRET experimental layout and data collection. In addition, there is a discussion of possible challenges faced in data interpretation due to false positives and false negatives that can arise from adventitious interactions in vesicles [1, 7, 10], inefficient labeling efficiency of the molecules [10], or light scattering.

2 Materials

2.1 Solid-Phase Peptide Synthesis

1. Automated peptide synthesizer.
2. Amino acids.
3. Resin: Fmoc-PAL-PEG.
4. Activator: 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU).
5. Base: *N*-methylmorpholine (NMM).
6. Deprotecting agent: 20 % Piperidine.
7. Solvent: Dimethylformamide (DMF), Dichloromethane (DCM), 1-methyl-2-pyrrolidone (NMP).

2.2 N-Terminal Labeling with Fluorophores

1. Fluorophore for solid-phase coupling.
2. Solvent: DMF, NMP.
3. Base: *N*,*N*-Diisopropylethylamine (DIPEA).
4. Activator: Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP).

2.3 HPLC Purification and Mass Spectrometry (MS)

1. Reversed phase semi-preparative and analytical column.
2. HPLC instrument.
3. Solvents: Acetonitrile, Isopropanol, water, Trifluoroacetic acid (TFA).
4. Lyophilizer maintained below −80 °C.
5. Glassware: glass screw cap vials, pear-shaped glass flask.
6. Compressed N_2 gas.
7. Mass spectrometry facility.

2.4 Solubilization of TM Peptides in Lipids and FRET Measurements

1. Solvents: Hexafluoroisopropanol (HFIP), Chloroform, Trifluoroethanol (TFE).
2. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid (Avanti Polar Lipids).
3. Purified TM peptides.
4. Compressed N_2 gas.
5. Liposome buffer (10 mM Sodium Phosphate Buffer, 500 mM NaCl, pH 7.0).
6. Dry ice, acetone, 37 °C water bath.
7. TFE lined glass screw cap vials, glass syringes.
8. UV/Vis spectrophotometer, quartz cuvettes.
9. Plate reader or Fluorimeter (If using a plate reader, the plate reader should contain a monochromator, such as the Tecan M1000 which provides for a Fluorescence intensity scan instead of point measurements from fixed emission wavelength filters).

3 Methods

3.1 Fmoc Solid-Phase Peptide Synthesis

1. Review the amino acid sequence of the peptide/peptides of interest, adding positively charged Lys residues to increase aqueous solubility of the highly hydrophobic sequence (*see* **Note 1**) [11].
2. Decide the scale of synthesis and the resin to be used (*see* **Note 2**). Weigh the calculated amount of resin, use the appropriate solvent (DMF/NMP) and activator (HATU), and set up the automated synthesis. A review of solid-phase peptide synthesis should be consulted for standard protocols and optimizations (*see* **Note 2**) [12].
3. Take the resin containing the completed peptide, check for correct sequence by MS (*see* **Note 3**), and then proceed with on-resin N-terminal labeling of the peptide with the fluorescent dye (*see* **Note 4**).

   This step onward, perform all experiments protected from light, till the end of the chapter.

3.2 N-Terminal Labeling with Fluorescent Dye

1. Divide the resin with peptide in half and proceed with two separate manual N-terminal couplings using donor and acceptor fluorescent dyes comprising a good FRET pair. If the dye is not carboxylic acid derivatized to enable Fmoc chemistry on a deprotected amine group, then an appropriate linker is attached to the amine group first (e.g., aminohexanoic acid, mini-PEG), followed by attachment of the fluorescent dye.

2. Use DMF and NMP as solvents and PyBOP/PyAOP as activators using the protocol optimized for hydrophobic sequences [13] (*see* **Note 5**).
3. Cleave the labeled peptide, precipitate using cold ether, and dry it under a stream of compressed N_2 gas with agitation by a glass rod to avoid clumping.
4. Blanket the peptide with a stream of N_2 or Ar, seal the cap with parafilm, and store it at −20 °C.

3.3 Reversed Phase HPLC Purification

1. Solubilize a small amount of the peptide in the appropriate solvent for HPLC purification (*see* **Note 6**). Blanket the rest of the peptide with a stream of N_2 or Ar, seal the cap with Teflon, and store it in −20 °C.
2. Filter the sample with a 0.22 μm filter.
3. Using the appropriate column for use, inject the sample and follow a gradient of 2–100 % Buffer B in 98 min (1 % per minute). Monitor the chromatogram at 280 nm if Trp or Tyr is present and at the wavelength of the fluorescent dye (*see* **Note 7**).
4. Collect the various fractions and analyze them using MS to identify the peak of interest (*see* **Note 7**).
5. Optimize the gradient to achieve better separation and collection of individual peaks.
6. Perform multiple HPLC runs using the same method and pool in the desired fractions from the various runs in a glass pear-shaped flask of appropriate volume such that the total volume doesn't exceed a third of the flask.
7. Flash-freeze the sample in the flask, rotating it over liquid N_2 with a tilt to increase the surface:volume ratio for better lyophilization. Attach it to the lyophilizer, cover it with foil, and leave it overnight till it forms a dry powder.
8. Take the flask out of the lyophilizer and empty its contents into a pre-weighed glass vial with screw cap. Weigh the vial with the sample in it and note the weight of the final sample.

3.4 Solubilization of Peptides and Lipids to Make Stock Solutions

1. Dissolve 1 mg of powdered POPC lipid (Avanti Polar lipids) in 1 mL of 1:1:1 HFIP:Chloroform:TFE in a screw cap glass vial and keep it tightly sealed. Calculate the number of moles/μL according to Scheme 1.
2. Turn on the spectrophotometer and set up the method for protein concentration scan including the absorbance wavelength of Trp (280 nm) and that of the fluorescent dyes.
3. Perform a blank scan with 1:1:1 HFIP: Chloroform: TFE (solvent).

Molecular weight = 760.10g/mol

No. of moles = weight/molecular weight

$$= \frac{1\text{mg}}{760.1\text{mg/mol}}$$

= 1.31 umol in 1 ml, or 1.31 nmol/ μL

Scheme 1 Calculation for number of moles for 1 mg/mL POPC lipid stock

Concentration of the fluorescent label on peptide:

$$[\text{Fluorophore}] = A_{max}/\varepsilon_{Fluorophore} l$$

where A_{max} is absorbance at λ_{max} of the dye
$\varepsilon_{Fluorophore}$ is extinction coefficient of the dye at that absorbance
l is path length of the cuvette (usually 1cm)

Concentration of the peptide based on Trp absorbance (λ=280nm)

$$[\text{Peptide}] = A_{280} - (A_{max} * CF)/\varepsilon\, l$$

where A_{280} is absorbance at 280nm
CF is the correction factor that adjusts for absorbance at 280 nm by the fluorophore, and is given by A_{280}/A_{max}
ε is extinction coefficient of the peptide (calculated based on no. of Trp, Tyr and Cys)

After calculating the concentration in terms of Molarity, calculate the number of moles in the stock solutions of the peptides similar to Scheme 1.

Calculating the labeling efficiency

$$\text{Percent labeling} = [\text{Fluorophore}]/[\text{Peptide}] * 100$$

Note: For peptides that do not have a Trp or Tyr to get accurate concentration from UV/VIS Spectrophotometry, CD can also be used to determine peptide concentration(3)

Scheme 2 Calculation of protein concentration and labeling efficiency

4. Scoop out a tiny amount of donor-labeled peptide and add it to 100 μL of the solvent. Label this as 'stock 1'.
5. Make a 1:10 dilution of stock 1 (call it 'stock 2') and add it to the appropriate cuvette for protein concentration scan (*see* **Note 8**).
6. Calculate concentration of the peptides and the labeling efficiency according to Scheme 2 (*see* also Subheading 3.8).
7. Repeat **steps 4–6** for the acceptor-labeled peptide.

3.5 Setup of the Peptide:Lipid Ratios for FRET

The goal of this experimental setup is to achieve a large concentration range of peptide or a range of peptide:lipid ratios which spans the spectrum from a "no FRET" sample to a "maximum FRET" sample. Since liposomes, unlike detergent micelles, do not

"communicate" with other liposomes to exchange peptide molecules, titrating the solution with more lipids will not dilute the peptide in the lipid solvent, or change the effective mole fraction of the peptides in the liposomes. Thus to obtain sufficient number of data points for an accurate curve fitting to obtain the energetics of the system, samples spanning a wide range of peptide:lipid ratios have to be prepared in separate tubes. Lipids can be equilibrated to form multilamellar vesicles (MLVs) as described later. It has been shown that FRET efficiencies for helix–helix interactions in large unilamellar vesicles (LUVs) are comparable to that of MLVs, and thus this protocol follows setting up FRET interactions in MLVs [3].

1. Once the peptide stocks for donor- and acceptor-labeled peptides and the lipid stocks are ready, start labeling small 12×35 mm screw cap vials for the FRET experiments.
2. Cover the labeling on the tubes with a tape to prevent the labels from being washed away by acetone in the dry-ice bath used in future steps.
3. From the peptide stock solutions, calculate the volume required for 50 pmol of the donor- and acceptor-labeled peptides (*see* **Note 9**).
4. Take a corning black 96-well plate, and add the required volume for 50 pmol of the donor peptide, say 8.9 μL, into one well. If the well volume is 75 μL, add $75 - 8.9 = 66.1$ μL of liposome buffer into it and mix by pipetting up and down a few times. In another well, do the same for the acceptor peptide. In a third well, add 75 μL of liposome buffer only.
5. Take the plate to the plate reader and run two separate fluorescence scans—one spanning the absorbance spectrum of the donor and another of the acceptor (*see* **Note 10**).
6. For the "donor-only" well, set the excitation maximum wavelength of the donor such that there is maximum overlap between the donor emission and acceptor excitation spectrum. If the emission spectrum looks noisy, try changing the parameters of the instrument (gain, PMT voltage, slit width, etc.) or increase the amount of peptide to get a smooth signal. The goal is to achieve a high enough concentration of the peptide for good signal to noise ratio (*see* **Note 11**).
7. Again, for the "acceptor-only" well, set the same excitation wavelength used in **step 6**. This step is to test for spectral bleedthrough between the donor and acceptor pairs. A minimal emission peak at the acceptor emission maxima on being excited by the donor excitation maxima is a sign of a good FRET pair.
8. Perform both **steps 5** and **6** for the "liposome buffer" well. This sample serves as a blank for data analysis.

9. At this point, start making the peptide:lipid ratio calculations. An example of the volumes is given in Table 1, based on the example in Subheading 3.4, **step 4**.
10. Pipette these solutions using glass syringes into the small glass screw cap vials arranged in increasing peptide:lipid ratio on a vial rack and vortex vigorously to mix them (Fig. 3a).
11. Take each vial in a fume hood and slowly evaporate the solvent using a light stream of compressed N_2 gas till all the solvent has evaporated, leaving a thin whitish lipid film at the bottom/sides of the vial (Fig. 3b) (*see* **Note 12**).
12. Don't screw the caps on the vials. Cover the tray with vials with aluminum foil to protect it from light, and pierce holes on the foil at the mouth of each vial.

Table 1
Sample 15 with the highest amount of lipid and same amount of unlabeled peptides serves as a scattering control to subtract any background scattering from the lipids alone

Sample No.	Mole fraction (peptide:lipid ratio)	Volume of donor stock (μL)	Volume of acceptor stock (μL)	Volume of 1 mg/mL POPC lipid stock (μL)
1	1:100	Calculate for 50 pmol (8.9 μL)	Calculate for 50 pmol	Calculate for 10 nmol (=7.7)
2	1:200	Ditto	Ditto	20 nmol = 15.4
3	1:500	Ditto	Ditto	50 nmol = 38.5
4	1:1,000	Ditto	Ditto	100 nmol = 77
5	1:2,000	Ditto	Ditto	200 nmol = 154
6	1:5,000	Ditto	Ditto	500 nmol = 385
7	1:10,000	Ditto	Ditto	1 mmol = 770
8	Control 1	Ditto	x	10 nmol = 7.7
9	Control 2	Ditto	x	20 nmol = 15.4
10	Control 3	Ditto	x	50 nmol = 38.5
11	Control 4	Ditto	x	100 nmol = 77
12	Control 5	Ditto	x	200 nmol = 154
13	Control 6	Ditto	x	500 nmol = 385
14	Control 7	Ditto	x	1 mmol = 770
15	No fluorophore control (contains 100 pmol unlabeled peptide)	x	x	1 mmol = 770
16	No liposome control	Ditto	Ditto	x

Sample 16 serves as a control for any FRET arising out of peptide aggregates in solution in the case of incomplete incorporation of peptides into vesicles

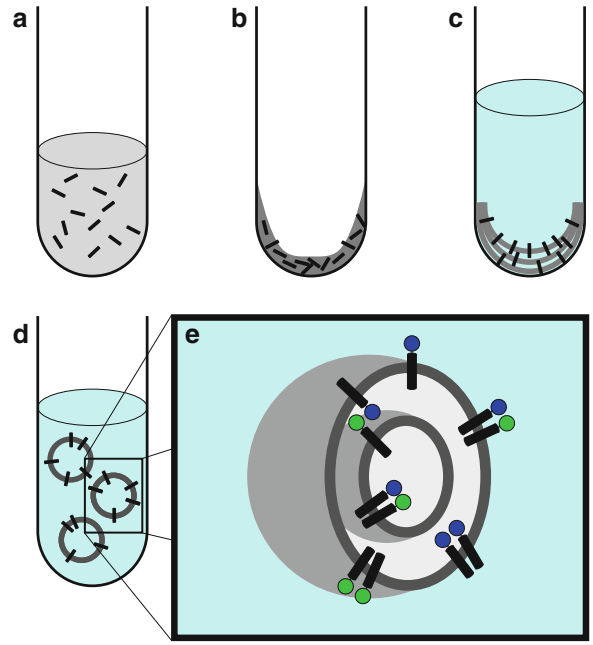

Fig. 3 Setup of FRET in liposomes. (**a**) Peptides and lipids dissolved in solvent and mixed together in a glass vial. (**b**) The solvent is evaporated using a stream of Nitrogen gas to leave behind a thin lipid film containing the peptide molecules. (**c**) The lipid film is hydrated using aqueous buffer, vortexed, and freeze–thawed to achieve proper equilibration and (**d**) formation of multilamellar vesicles containing the peptides. (**e**) The various pairs that will be contributing to FRET efficiency due to association of peptides and just colocalization, as well as "dark pairs" formed by donor–donor, acceptor–acceptor associations

13. Place the covered tray inside a vacuum dessicator overnight (*see* **Note 13**).
14. The next day, add 75 μL (or 100 μL depending on the volume of the wells in the plate or the cuvette of the fluorimeter) of liposome buffer into each vial, screw its cap on tightly, and vortex vigorously for about a minute (Fig. 3c). The samples with higher lipid will turn more turbid. Note the extent of turbidity in these samples.

3.6 Preparation of Multilamellar Lipid Vesicles (MLVs) Using Freeze–Thaw Cycles

1. Prepare a dry-ice/acetone bath and a water bath set at 37 °C.
2. Place the tray with vials on the dry-ice/acetone bath for about 1 min. Make sure all the samples are frozen and the labels are not getting washed off.
3. Now place the tray on the water bath, shaking them mildly. A crackling sound will signify thawing of the liquids. Continue thawing till the sound subsides.
4. Repeat **steps 2–3** alternately for 2–3 cycles.

5. Now note the turbidity of the samples again. Decrease in turbidity signifies formation of MLVs (*see* **Note 14**). At this point, the donor and acceptor peptides should have equilibrated with the lipid and with each other based on their affinity and the peptide-to-lipid ratio (Fig. 3d).
6. Pipette out the samples from the vials into a 96-well corning plate according to Table 1. Do not pipette up and down to avoid formation of air bubbles in the sample as they can lead to light-scattering effects.

3.7 Measurement of FRET and Data Analysis

1. Take the plate to the plate reader, insert the plate, and adjust the settings for the plate (or alternatively use a fluorimeter).
2. Set the excitation wavelength as that for maximum spectral overlap for the pair and collect the fluorescence emission scans spanning the emission wavelengths of both the donor and acceptor fluorophores. Maintain the same scan for each sample.
3. The "Control" samples in Table 1 serve as the "no FRET" controls for their corresponding sample row. For instance, sample 1 in Table 1 has a 1:100 peptide-to-lipid ratio where there are 50 pmol of donor and acceptor each. Control 1 therefore has 50 pmol of the donor only, with the same amount of lipid as sample 1.
4. Calculate percent FRET, $E(\%)$, by the wavelength of the emission maximum of the donor in the absence and presence of the acceptor according to Scheme 3.

3.8 Discussion

A lot of factors need to be taken into account for accurate FRET intensity measurements. One of the most important factors is the labeling efficiency of peptides. Hydrophobic peptides pose a greater level of difficulty for their labeling and purification, and alternative methods for labeling have been discussed [13]. Once this step has been optimized, the next level of difficulty arises in accurate quantification of these peptides. An unlabeled peptide is commonly quantified using UV/Vis spectrophotometry where the Trp and Tyr absorbance at 280 nm is measured and protein concentration determined using Beer's law. In the case of a labeled peptide, the absorbance of the peptide sample at 280 nm comprises three components:

1. Trp/Try absorbance of labeled peptide
2. Trp/Tyr absorbance of unlabeled peptide
3. Absorbance of the dye at 280 nm

Thus it is important to differentiate the individual components for accurate quantification. This is done using the "Correction Factor of the dye" and the labeling efficiency of the peptide

$$E_{observed} = [(I_D - I_{DA})/ I_D] \quad \text{...Eq. (1)}$$

where I_D nd I_{DA} are the donor emission maximum intensities of samples containing only donor-labeled proteins (controls)and samples with both donor-and acceptor-labeled proteins, respectively.

$$E_{expected} = \frac{[D][A]}{[D][D]+[D][U]+[D][A]}$$

where $[D] = [D]^{total} * L^D$

$[A] = [A]^{total} * L^A$

and $[U] = [D]^{total}(1-L^D)+[A]^{total}(1-L_A)$

($[D]$is concentration of labeled donor-peptide, L_D is labeling efficiency of donor-peptide and $[D]^{total}$ is total donor-peptide, $[A]$is concentration of labeled acceptor-peptide, L_A is labeling efficiency of donor-peptide and $[A]^{total}$ is total acceptor-peptide, and $[U]$is concentration of unlabeled peptide)

$$\textbf{Fraction Dimer} = E_{observed} / E_{expected}$$

$$= \textbf{Dimer / Total Peptide}$$

$$= 2X_{dimer} / (2X_{dimer} + X_{monomer}) \quad \text{...Eq. (2)}$$

where X is the mole fraction of the peptide.

Finally, mole fraction concentrations can be used to calculate a partition coefficient by

$$K_x = [X_{dimer}]/ [X_{monomer}]^2 \quad \text{...Eq. (3)}$$

which is an equilibrium constant, and the free energy of association is calculated by

$$\Delta G_x = -RT \ln (K_x)$$

Scheme 3 Calculation of percent FRET efficiency

(Scheme 2). However, to be able to use the calculations in Scheme 2, another important parameter needs to be established, the molar extinction coefficient, $\varepsilon_{\text{Fluorophore}}$ *in the given solvent system*. Vendors selling dyes usually provide the molar extinction coefficient values of dyes at their A_{max} in an aqueous buffer at a particular pH. But these values of $\varepsilon_{\text{Fluorophore}}$ and A_{max} significantly change in different solvents (Fig. 4). For accurate determination of equimolar ratios of the peptides, it is very important to characterize the behavior of the fluorophores in the solvent system being used. This can be done by plotting a calibration curve of the A_{max} of the dye in the given solvent versus concentration. Sometimes it is difficult to dissolve a known amount of dye in the organic solvent at a measurable concentration. In that case it is advisable to make the stock solution in a buffer that it has been characterized in, and then use the provided molar extinction coefficient and λ_{max} values to calculate the stock concentration. One should make this concentration high enough such that a very small volume can be used to dilute into the organic solvent to obtain the calibration curve. Then serial

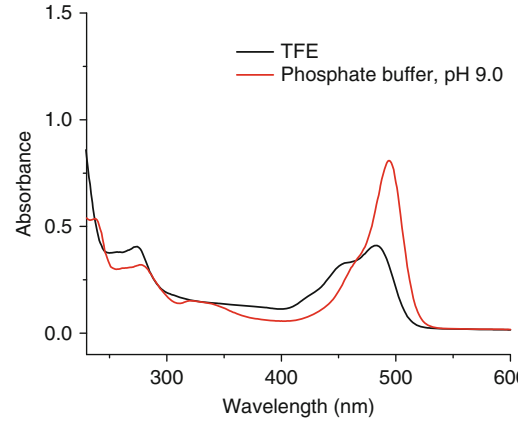

Fig. 4 Absorbance spectra of FITC in Phosphate Buffer, pH 9.0, and in TFE. The solvent has a blue shift effect on the λ_{max} and its molar extinction coefficient ε

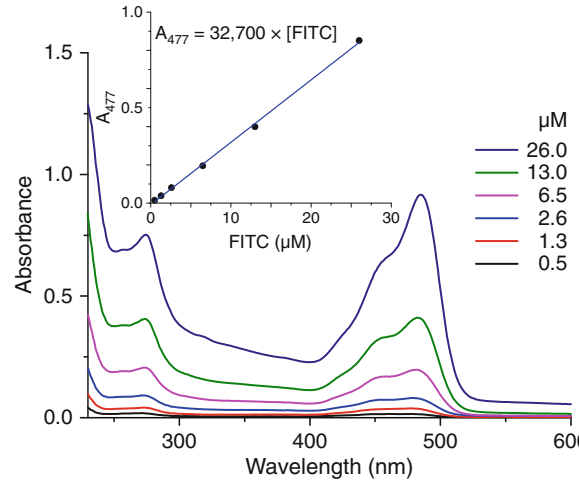

Fig. 5 Calculating the molar extinction coefficient $\varepsilon_{Fluorophore}$ of FITC in TFE. Absorbance scans of increasing concentrations of FITC dye in organic solvent. A_{max} (477 nm) is plotted versus concentration and the slope of the curve yields the new molar extinction coefficient $\varepsilon_{Fluorophore}$ of FITC (32,700 M^{-1} cm^{-1}) in TFE (*inset*). The ratio of A_{280} to A_{max} yields the new correction factor for FITC in TFE required for calculating the degree of labeling. Note that the wavelength of maximum absorbance shifts and that the molar extinction coefficient at A_{max} decreases more than twofold compared to that in Phosphate Buffer pH 9.0

dilutions should be made to obtain samples of known concentration in organic solvent, absorbance scans should be taken (Fig. 5), and A_{max} of the dye in that solvent versus concentration should be plotted (Fig. 5 inset). The slope of the curve will provide for the $\varepsilon_{Fluorophore}$ at the new λ_{max} of the dye in the organic solvent. Once the behavior of the dye has been characterized in the given solvent in this manner, the concentration and labeling efficiency of the peptide can be

calculated according to Scheme 2 and utilized in Scheme 3 to calculate the FRET efficiency values.

Apart from labeling efficiency, another contributor towards the observed FRET intensity is random colocalization due to proximity of donors and acceptors, which needs to be subtracted from the steady state FRET observed. Depending on the level of quantification necessary for the experiment at hand, there are several ways by which this can be taken into account [14, 15]. The peptide pairs that will be forming in a self-associating system of TM peptides will be the random proximity pairs, along with *DA*, *DD*, *AA*, *DU*, *AU*, and *UU* (where *D* is donor-labeled peptide, *A* is acceptor-labeled peptide, and *U* is unlabeled peptide). Figure 3e shows a schematic representation of the possible pairs. Finally, once all these parameters have been characterized, the FRET efficiency can be calculated according to Scheme 3. Contributions of proximity and sequence-dependent association to FRET efficiency can be distinguished by spiking the existing donor–acceptor-labeled samples with unlabeled peptide at the time of preparation. If the FRET efficiency is primarily due to sequence-dependent association, then addition of unlabeled peptides will lead to a decrease in the FRET efficiency [3, 7].

For a homodimer, the FRET efficiency, a measure of association, (Scheme 3, Eq. (2)) is calculated according to Scheme 3, Eq. (1), and the data is fitted to a curve using Scheme 3, Eq. (3) to yield the association constant and the free energy values [3, 7].

4 Notes

1. Lysine tags increase solubility and decrease peptide aggregation in hydrophobic sequences by providing for unfavorable electrostatic interactions [11]. The number of Lys residues incorporated and their positioning varies depending on the TM peptide sequence, and a detailed description of this optimization can be found in [11]. Addition of extra amino acids will also provide for a flexible linker between the peptide and the dye with which it will be labeled. It is important, however, to make sure that the linker length keeps the fluorophores within the limit of their Förster radius in all orientations.
2. It is advisable to begin with a smaller scale of synthesis as hydrophobic peptides usually need some optimization of the protocol for synthesis. A 25 μmol scale is a good starting point and is enough for preliminary experiments. Choice of resin is important—a low load resin (e.g., Fmoc-PAL-PEG resin from Applied Biosystems, with a 0.18 mmol/g loading capacity) will decrease aggregation potential of the peptides on the resin. After an assessment of the hydrophobicity of the final sequence, a “brute force” method can be applied for the preliminary trial.

For instance, in a Val-Val-Thr-Ala-His sequence, the difficult couplings are Val-Val, Val-Thr, and Thr-Ala. For these couplings, one could use double or triple coupling and extended coupling times, whereas for the Ala-His coupling, a single coupling should suffice. This approach has been utilized before [16] and is found to be effective for particularly hydrophobic sequences.

3. CHCA (4-Chloro-α-Cyanocinnamic Acid) is one of the best universal matrices for performing MALDI-TOF in peptides [17]. Typically a barely visible amount of peptide is dissolved in about 100 μL of Acetonitrile in water (percentage depending on solubility of peptide). The matrix can be prepared as follows: 10 mg of CHCA matrix in 1 mL of 50:50 Acetonitrile: water with 0.1 % TFA. Then various ratios of the matrix:peptide solutions are made and 1–2 μL of the final samples are spotted on the target for MALDI-TOF analysis.
4. For FRET, the peptide has to be labeled with a donor or acceptor molecule. In this procedure, the labeling will be done on the N-terminus manually, on-resin, using commercially available carboxylated fluorescent dyes. An important point is to program the automated synthesis to terminate the last coupling step before deprotection. This will keep the final N-terminal residue Fmoc protected and prevent unwanted reactions till the peptide is ready for labeling. Also, it is advisable that the peptide on-resin remains solvated till the next step to reduce aggregation. Before proceeding with the labeling process, it is better to take out a tiny amount of the resin (a touch with the spatula), deprotect it and cleave it using standard procedures [12], and then analyze it using MS. If the peptide has been synthesized correctly, deprotect the last amino acid on the entire resin manually and proceed with the labeling.
5. For quantitative FRET experiments, it is important to obtain high labeling yields of peptides, as the separation of the labeled from the unlabeled species in the case of hydrophobic peptides is difficult by HPLC [3, 7, 18–21]. Standard on-resin N-terminal labeling yields have been found to be very low for hydrophobic peptides, and thus a method for higher labeling efficiency of hydrophobic peptides was developed, utilizing larger amounts of dyes and coupling agents [13].
6. Dissolve minimum amount of peptide (~0.5 mg) in minimum amount of DMF. If the peptide does not readily dissolve, try other solvents like HFIP, THF, and TFE. Then slowly add water dropwise, till the peptide just starts precipitating, turning slightly murky. It is important to have a high enough percentage of water in the solution (≥ 60 %) for the peptide to bind the reversed phase column, but also maintain solubility of the peptide at the same time. If using TFA for solubilization, do not exceed the final TFA concentration to be above 5 %.

7. The Reversed Phase HPLC system usually consists of an *n*-alkylsilica-based sorbent from which the solutes are eluted. The most commonly used *n*-alkyl ligand is C-18, but for hydrophobic peptides, C2, C4, C8, phenyl, and cyanopropyl ligands provide better separation [22]. It is good practice to first perform a blank run with 100 % Buffer A till a stable baseline is obtained and then a blank run with the solvent the sample is dissolved in, before injecting the sample. Choice of columns for hydrophobic peptides varies and there are various parameters that can be changed for optimization. Usually a small volume (~10 μL) of the sample is injected into an analytical column first and analyzed for the retention time of the sample elution with a linear gradient from 2 % to 100 % Buffer B (Acetonitrile with 0.1 % TFA) over 30 min. For hydrophobic peptides which do not elute easily, sometimes using 60 % Isopropanol in Acetonitrile with 0.1 % TFA can be used for Buffer B.

   If LC-MS facilities are available, it is better to perform a run on an analytical column and monitor the masses of the peaks as the elute. The conditions can then be transferred to a semi-prep column having the same *n*-alkyl silica group for fraction collection.

8. Sometimes it is necessary to make higher dilutions of the initial stock solution of the peptide for accurate concentration determination. In that case, make serial dilutions till you get an absorbance value between 0 and 1, use the most diluted stock for concentration determination, and calculate the concentrations of the original stock by scaling up. Later in the experiment this stock will be used for solubilization with lipid and then involve evaporation of the solvents, and thus it is easier to have a higher stock concentration to minimize the volume of solvent to be evaporated.

9. If the stock concentration of the peptide is 0.2 mg/mL and you have 500 μL of that solution, the concentration in terms of number of moles is 5.6 pmol/μL. Thus, to use 50 pmol of peptide, use 50/5.6 = 8.9 μL of the stock solution. Calculate the stock concentration of each donor- and acceptor-labeled peptide in number of moles per microliter and start with a total of 100 pmol of 1:1 peptide (donor/acceptor) to begin the peptide/lipid molar ratios.

10. A fluorimeter can also be used in place of a plate reader, and the sample volumes in that case have to be according to the volume of the fluorimeter cuvette (~10–20 μL higher) than the cuvette volume to prevent the beam from hitting the meniscus of the solution leading to scattering. It has been shown that volume of the sample does not change the FRET efficiency as long as the amounts of lipid and peptide are the same [3].

11. It should be kept in mind that increasing the peptide amount will also mean increasing the amount of lipid to maintain a peptide:lipid ratio. If the lipid concentration is too high, light scattering by the lipid will interfere with the FRET results, so a balance has to be achieved. For a true scattering control, use the same amount of unlabeled peptide in the highest amount of lipid being used in the experiment to check for any scattering.
12. It may take ~20 min for vials with higher volumes of solvent to evaporate. The higher the lipid content, the longer will it take to evaporate and the more visible will the lipid film be.
13. This step can also be substituted by adding the samples in a lyophilizer for 2–3 h if the experiment has to be finished on the same day.
14. Freeze–thaw cycles are generally required for equilibration of buffers, salts, etc. MLVs, prepared by premixing the proteins and lipids in organic solvent and hydrating the mixture, are in equilibrium after a single freeze–thaw cycle [3]. When thin lipid films are hydrated, stacks of liquid crystalline bilayers become fluid and swell as shown in Fig. 3c. The hydrated lipid sheets detach during agitation and self-close to form MLVs preventing interaction of the hydrocarbon core of the bilayer from interacting with water at the edges. It has been shown that the presence of unlabeled peptide helps in equilibration of the peptides in the lipids and leads to a faster decrease in turbidity upon just one freeze–thaw cycle.

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