Chapter 27

Measuring ECS Interaction with Biomembranes

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Abstract

Understanding the correct interaction among the different components of the endocannabinoid system (ECS) is fundamental for a proper assessment of the function of endocannabinoids (eCBs) as signaling molecules. The knowledge of how membrane environment is able to modulate intracellular trafficking of eCBs and their interacting proteins holds a huge potential in unraveling new mechanisms of ECS modulation.

Here, fluorescence resonance energy transfer (FRET) technique is applied to measure the binding affinity of ECS proteins to model membranes (i.e., large unilamellar vesicles, LUVs). In particular, we describe in details the paradigmatic example of the interaction of recombinant rat FAAH-ΔTM with LUVs constituted by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).

Key words Biological membranes, Membrane proteins, Large unilamellar vesicles, FRET, Binding isotherms, Membrane affinity

1 Introduction

Membrane proteins interact preferentially with a specific lipid environment, and the enrichment of specific lipids around a protein can dramatically modulate its functional activity [1, 2]. Many cell functions are governed by the peculiar organization of lipids and proteins within biological membranes. Membrane fusion, intermembrane exchange of lipid components, formation of lipid domains in lipid mixtures, and vesicle-vesicle interactions all involve changes in the distances between different lipid components.

The plasma membrane bilayer is a closely packed and crowded environment, and fluorophores, behaving as donor and acceptor molecules with good spectral overlap, can communicate by FRET over lateral distances that span several lipid molecular diameters. FRET is one of the most useful techniques to interrogate these processes. Another major application of FRET in cell biology is the analysis of protein-protein and protein-lipid interactions.

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These are often important for intercellular communication and for successful transfer of information within a cell, leading to specific signal transduction pathways. FRET is also a useful tool for measuring both average distances of proteins from membranes and peripheral binding of proteins and other molecular components to the membrane surface [3].

FRET is a process whereby the energy of an excited state is non-radiatively transferred from an excited donor molecule to an acceptor molecule [4]. For FRET to occur, a number of conditions need to be fulfilled: (a) the emission spectrum of the donor needs to have sufficient overlap with the absorption spectrum of the acceptor; (b) the emission and absorption dipole moment of donor and acceptor must not be perpendicular; (c) donor and acceptor have to be in close proximity [4].

Förster [5, 6] was the first to establish that energy can be transferred efficiently by a resonance dipole-dipole mechanism over distances from 10 to 100 Å. Since these pioneering studies, FRET has been successfully used in different fields of research for over 40 years. The increasing availability of new synthesized/purified biological macromolecules, along with the increasing availability of fluorescent compounds, will continue to facilitate the design of innovative biotechnological applications of FRET.

One of the classical and most prevalent uses of FRET is to measure distances between defined sites within a protein structure and to study its oligomeric organization in solution. Indeed, proteins contain intrinsic fluorophores (i.e., Trp and Tyr), and they can also be covalently labeled (often specifically) with extrinsic fluorophores. Many of these extrinsic probes are commercially available and easy to use.

With the Förster equation it is possible to calculate the efficiency of the energy transfer that occurs between the donor fluorophore and the acceptor fluorophore, and the distance at which it may occur [5, 6]. However, the Förster equation can be used only if there is a single donor-acceptor pair, because the presence of more than one donor, or of more acceptors, reduces the accuracy of data acquisition. Thus, we used FRET to describe a protein-lipid interaction where the donor is a constituent of the protein (Trp), with the emission maximum from 307 to 353 nm, and the acceptor fluorophore is the synthetic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl) (PyPE), with absorption maximum at 351 nm [7, 8]. Of note, the latter compound can be embedded within synthetic membranes. In this setup, analysis of the FRET signal allows to monitor quenching of Trp fluorescence that occurs at different concentrations of vesicles containing the acceptor fluorophore (Fig. 1). From these data, binding isotherms are obtained to calculate membrane affinity of the investigated protein.

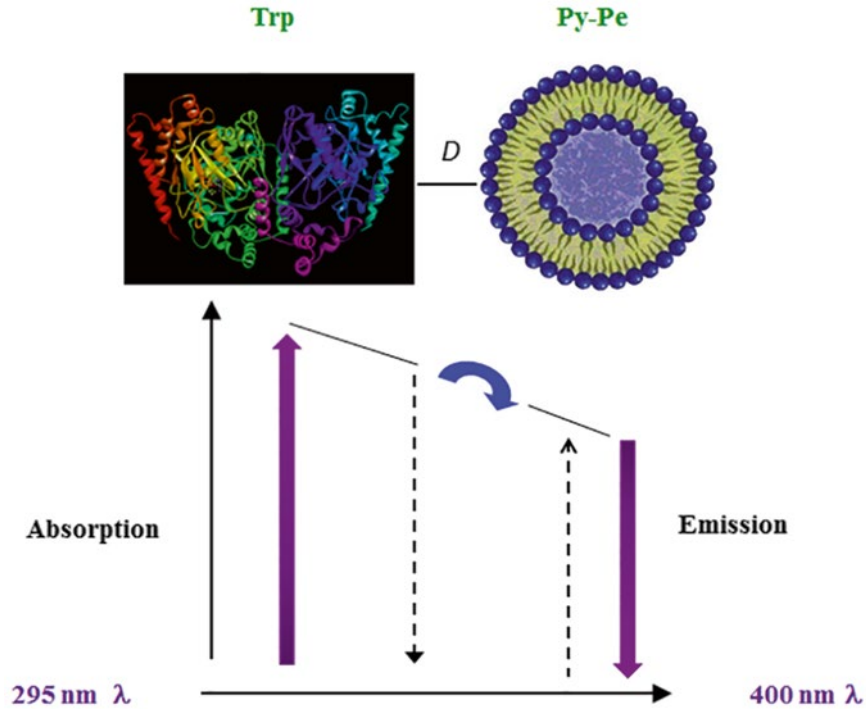

Fig. 1 Scheme of the energy transfer in a typical FRET experiment. *Trp:* intrinsic protein tryptophan donor fluorophore, *PyPE:* acceptor fluorophore embedded within the liposome bilayer, *D*: Förster distance

2 Materials

Use reagents of high analytical grade, and prepare buffers with ultrapure water.

2.1 Components for Liposome Preparation

Glass test tubes (2 cm diameter).
Ethyl alcohol (gradient grade) ≥99.8 % pure.
Chloroform.
POPC: 16:0–18:1 PC.
PyPE:18:1pyrenePE1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl), ammonium salt (*see* **Note 1**).

Buffer: 50 mM Tris–HCl, pH 7.5. In a glass beaker, weigh 6.06 g Trizma base, add 800 ml of water, and adjust pH to 7.5 with concentrated HCl. Bring to a final volume of 1 l with water in a volumetric flask. Store at 4 °C.
Nitrogen gas.
Water bath.
LiposoFast apparatus, equipped with 100 nm pore-size polycarbonate membranes and gastight syringes (500 μl volume).

2.2 Components for Isolation of Rat Liver Membranes

Potter glass (5 ml).
Protease inhibitor cocktail (IC).

Buffer A: 5 mM Tris–HCl, 10 mM KCl, 5 mM EDTA, pH 7.8. In a glass beaker, weigh 0.606 g Trizma base, 0.74 g KCl, and 1.9 g EDTA. Add 800 ml of water, adjust pH to 7.8 with concentrated HCl, and bring to a final volume of 1 l with water in a volumetric flask. Store at 4 °C.

Buffer B: Buffer A containing 1 mM DTT and IC. In a glass beaker, weigh 0.5 mg DTT and 30 μl of IC, and bring to a final volume of 3 ml with Buffer A in a volumetric flask.

Buffer A 60 % sucrose: In a glass beaker, weigh 60 g sucrose and bring to a final volume of 100 ml with Buffer A in a volumetric flask. Store at 4 °C.

Buffer A 37 % sucrose: In a glass beaker, weigh 37 g sucrose and bring to a final volume of 100 ml with Buffer A in a volumetric flask. Store at 4 °C.

2.3 Protein Solution

Recombinant rat FAAH-ΔTM is dissolved in the same buffer used for liposome preparation. Use only highly purified protein samples. Enzyme purification is a complex procedure [10, 11] that remains far from the scope of this chapter. The reader can obtain pure FAAH-ΔTM through collaboration with our group or others.

2.4 FRET Components

LS50B fluorimeter (PerkinElmer Life and Analytical Science, Boston, MA) connected to a circulating water bath. Adjust excitation and emission slits to 4 nm and 10 nm, respectively, with a scanning speed of 1500 nm/min (*see* **Note 2**).

Quartz cuvettes with a maximum volume of 250 μl (*see* **Note 3**), and a light path of 10 mm.

3 Methods

3.1 Liposome Preparation

Warm about 100 ml of buffer at 25 °C in a water bath (*see* **Note 4**).

Dissolve 0.5 mg PyPE in 100 μl of chloroform, in order to have a 5 mg/ml PyPE solution (*see* **Note 1**).

To prepare 1 ml of liposomes composed of POPC at a concentration of 2 mM, weigh 1.5 mg POPC in a glass test tube and dissolve it in 200 μl of ethanol (*see* **Note 5**).

Take 100 μl of this solution and put it in a second test tube, together with 2 μl of 5 mg/ml PyPE solution. The final lipid:fluorophore molar ratio is 100:1 (*see* **Note 6**).

Evaporate the organic solvent in the two test tubes under a N_2 stream, keeping the tubes immersed in the water bath at a controlled temperature of 25 °C (*see* **Note 4**). Gently rotate the test tubes, in order to form a monomolecular film around the walls. Once the dry lipid film is formed, add 1 ml of warm buffer in each test tube while keeping it immersed in the water bath; this proce-

dure will facilitate the emulsion of the lipid and the correct formation of liposomes (*see* **Note 7**).

In order to have homogeneous POPC LUVs in size, use the extrusion method. To this aim, use the LiposoFast extruder immersed in a water bath at 25 °C. This instrument consists of two 500 μl syringes and a steel jacket containing a polycarbonate membrane with a 100 nm pore diameter, through which lipids are fluxed. Pushing a multilamellar lipid solution 11–21 times through the membrane will produce homogeneous unilamellar vesicles with lateral dimensions of about 100 nm. The formation of LUVs is macroscopically evident as clarification of the lipid mixture (*see* **Note 8**).

To investigate lipid composition-induced preferential binding of a protein, instead of liposomes consisting of POPC alone, LUVs

Table 1
Preparation of LUVs of defined concentration

	LUV concentration in stock solution (mM)	Added volume (μl)	Total volume (μl)	Final LUV concentration (μM)	Final protein concentration (μM)
0		0	100	0	0.2
1	0.4	1	101	4	0.2
2	0.4	1	102	8	0.2
3	0.4	1	103	11	0.19
4	0.4	1	104	15	0.19
5	0.4	1	105	19	0.19
6	0.4	2	107	26	0.19
7	0.4	2	109	33	0.18
8	0.4	1	110	36	0.18
9	0.4	1	111	40	0.18
10	0.4	4	115	52	0.17
11	0.4	4	119	64	0.17
12	0.4	2	121	70	0.17
13	0.4	4	125	80	0.16
14	0.4	5	130	92	0.15
15	0.4	5	135	103	0.15
16	2	4	139	158	0.14
17	2	4	143	210	0.14
18	2	4	147	258	0.14
19	2	4	151	305	0.13

can be produced with different lipid compositions, or with lipids extracted from specific cell membranes (Table 1).

For instance, LUVs from the plasma (PM) and the endoplasmic reticulum (ER) membranes can be prepared from lipids extracted from rat hepatocytes, as follows. Disrupt liver tissue by a Potter glass, and mix with a volume of Buffer B (*see* **Note 9**). After homogenization, liver is centrifuged at 10,000 × *g* for 5 min, supernatant (~2.4 ml) is transferred to a 14 ml ultracentrifugation tube, and 9.6 ml of Buffer A containing 60 % of sucrose (final sucrose concentration: 48 %) is gently overlayed with a Pasteur pipette. Separately, prepare two tubes with 1 ml of Buffer A, and two tubes with 4.5 ml of Buffer A containing 37 % of sucrose. Then, in a 14 ml tube add 6 ml of the previously prepared solution containing 48 % of sucrose, slowly stratify over 4.5 ml of Buffer A containing 37 % of sucrose, and finally add 1 ml of buffer A. In another tube, repeat the same procedure with the remaining 6 ml of the solution containing 48 % of sucrose. Then, centrifuge the tubes at 150,000 × *g* for 2 h. In the last step, recover PM and ER lipid membranes with a glass Pasteur pipette (*see* **Note 10**), then wash with Buffer A in a new tube, and centrifuge again at 150,000 × *g* for 1 h [9]. Recover the pellet containing the two different membranes, dissolve it in 200 μl of chloroform, evaporate the organic solvent under a N_2 stream, and proceed as described for the preparation of the liposomes.

3.2 Fluorescence Measurements

In the fluorimeter, set the excitation wavelength at 292 nm (Trp excitation) and record fluorescence emission spectra between 300 and 600 nm.

Record the spectrum of the blank (i.e., 100 μl of buffer), to be subtracted from all spectra (*see* **Note 11**).

Record the value of Trp fluorescence emission intensity (FI) at its maximum (approximately 340 nm). Note that the maximum of fluorescence emission of PyPE is about 380 nm (*see* **Note 12**).

To correctly perform the procedure, it is important to set up three series of measurements of FI maximum, according to the scheme below. Of note, the first series is done for the correction of FI due to protein dilution. The second series is for eliminating the eventual contribution of quenching Trp emission due to protein/lipid interaction. The third series is the measurement of FRET.

The first step (step 0) of each series is a measure of FI spectrum of the protein solution (F0), that is, 100 μl of 0.2 μM rat FAAH-ΔTM (*see* **Note 13**).

1. In the first series, add to 100 μl of protein solution in the quartz cuvette increasing amounts of buffer, as reported below in column 2, and record Trp FI maximum ($F_{(\text{protein})}$) (*see* **Note 14**).

2. In the second series, add the same volumes indicated below in column 2 of LUV_S from the diluted or concentrated stock solutions (column 1), and record Trp FI maximum ($F_{(lipid)}$).
3. In the third series, register Trp FI maximum upon addition of increasing amounts of LUVs containing the fluorophore ($F_{(lipid/PyPE)}$) (*see* **Notes 14** and **15**).

Do the additions directly into the cuvette and mix thoroughly. Let the sample equilibrate to the cuvette temperature for 5 min, before recording emission spectra.

For each series, normalize to 1 the value of F_0, and then normalize each FI value to F_0, in order to minimize the contribution of possible experimental errors in the determination of protein concentration.

For each measure (n), calculate the fluorescence intensity variation (ΔF) with respect to the maximum fluorescence intensity value:

$$\Delta F_n = F_0 - F_n \tag{1}$$

Subsequently, correct the normalized values of FI maximum for the effect of protein dilution and of lipid addition, in order to get ΔF values that are due to FRET, as follows:

$$\Delta F = \Delta F_{(lipid/PyPe)} - \left(\Delta F_{(lipid)} - \Delta F_{(protein)}\right) \tag{2}$$

where:

$\Delta F_{(protein)}$ = FI variation in the spectra due to the addition of buffer only (series 1).

$\Delta F_{(lipid)}$ = FI variation in the spectra due to the addition of the lipid (series 2).

$\Delta F_{(lipid/PyPe)}$ = FI variation in the spectra due to the addition of the lipid in the presence of the acceptor fluorophore (series 3).

ΔF is the fluorescence intensity variation at different lipid concentrations; therefore it follows that

$$\Delta F = f\left([\mathrm{L}]\right) \tag{3}$$

where [L] is the lipid concentration.

The protein binding isotherm to the lipid is described by the following function:

$$\Delta F = \left(\Delta F_{max}\,[\mathrm{L}]\right) / \left([\mathrm{L}]_{1/2} + [\mathrm{L}]\right) \tag{4}$$

By fitting the values of ΔF as a function of lipid concentration through nonlinear regression analysis of the binding isotherm with Eq. 4 (Fig. 2), ΔF_{max} and $L_{½}$ parameters can be obtained. ΔF_{max} is the maximum value of ΔF that is obtained under conditions of saturation of the protein-vesicle binding, and $[\mathrm{L}]_{½}$ is the concentration of lipid vesicles at which $\Delta F = ½\Delta F_{max}$ (Table 2) [13].

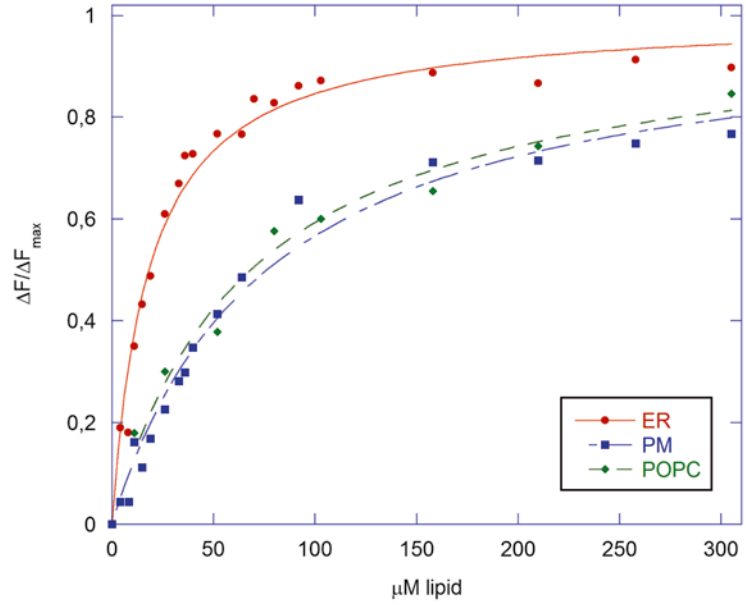

Fig. 2 Binding isotherms of rat FAAH-ΔTM as tryptophan FRET quenching at different concentrations of POPC (*green diamonds*), or of reconstituted LUVs from the ER (*red circles*) and PM (*blue squares*)

Table 2
$L_{½}$ values for the binding of rFAAH-ΔTM with membranes of different lipid compositions (modified from ref. 1)

LUV	$L_{1/2}$ (μM)
POPC	67 ± 10
Endoplasmic reticulum (ER)	18 ± 3
Plasma membrane (PM)	79 ± 8

4 Notes

1. Dissolve PyPE powder in organic solvent (chloroform) at a concentration of 5 mg/ml, prepare 100 μl aliquots, dry with N_2 flux, and keep at −20 °C until use.
2. The setup of the slits has to be optimized for each instrument.
3. We optimized the method for a quartz cuvette with a window volume of 100 μl and maximum capacity of 250 μl.
4. Temperature must be kept above the main transition temperature (T_m) of the lipid, thus favoring a high mobility degree of the acyl chains, and an easy flux through the pores of the extruder.

5. Try to keep the solvent volume as low as possible, in order to reduce the time needed for evaporation.
6. In the literature a molar ratio 98:2 is reported [8], but this value can be adjusted to fit instrument characteristics.
7. During the preparation phase of the liposomes, it is appropriate to coat the tube containing the lipid/fluorophore mixture with an aluminum foil, in order to avoid photobleaching of the fluorophore.
8. The LiposoFast-Basic apparatus produces unilamellar liposomes by the manual extrusion of a multilamellar liposome suspension through a polycarbonate membrane of defined pore size. The sample is passed through the membrane by pushing it back and forth between two syringes (0.5 or 1.0 ml syringes). In order to facilitate the passage of the lipid suspension through the syringes during the extrusion process, it is important that the LiposoFast-Basic apparatus is immersed in a water bath heated at a temperature higher than the T_m of the lipid used. To obtain a homogeneous solution, it is recommended to have a minimum of 11 steps to a maximum of 21 steps, always in odd [12]. This procedure will prevent that any residual impurity can contaminate the final preparation. In order to avoid any carryover contamination of PyPE, at the end of each preparation syringes and the entire LiposoFast-Basic apparatus have to be perfectly cleaned by several rinses with hot ethanol, followed by hot distilled water.
9. For a liver of ~0.74 g, use 1.5 ml of Buffer B. Homogenize the liver, filter with a gauze, and then centrifuge at 2,000×*g* for 5 min at 4 °C. Add to the supernatant 1.5 ml of Buffer B, homogenize, and centrifuge again at 10,000×*g* for 5 min at 4 °C.
10. After centrifugation at 150,000×*g* for 2 h, stratification of the membranes can be observed in the tube from top to bottom, as follows: the first white layer is an unspecific fatty mixture, the second layer is constituted by PM, and the third layer is constituted by RE. Pellets represent cellular debris, and should be discarded.
11. If the buffer has an isotropic signal along all emission spectra, fluorescence intensity at 600 nm must be subtracted from all of them.
12. Trp maximum of fluorescence intensity is at ~340 nm. It is possible that during the binding measurements a blue or a red shift of this peak is observed, which can be due to eventual membrane-induced aggregation or dissociation of the protein sample, or variation of the chemical environment of the emitting Trp intrinsic fluorophores.
13. Protein concentration depends on the number of Trp residues in its primary structure, their solvent exposition, and the characteristics of the instrument. As a general rule, adjust protein

concentration to have a value of ~800 AFU for the measure of F_0 (at a lipid concentration = 0).

14. It is strongly recommended to store all values in a datasheet for subsequent analysis (e.g., by using Microsoft Excel or KaleidaGraph software).
15. In our method, we use liposome concentrations ranging from 10 to 300 μM. Others [13] have reported binding isotherms with different lipid concentrations (up to 1 mM) for different human enzymes. The lipid concentration range must be adjusted according to the affinity of the protein to the membrane.

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