FRET in membrane biophysics: an overview

Luis M. S. Loura1,2* and Manuel Prieto3**

1 Faculdade de Farmácia, Universidade de Coimbra, Coimbra, Portugal
2 Centro de Química de Coimbra, Universidade de Coimbra, Coimbra, Portugal
3 Centro de Química Física Molecular and Institute of Nanosciences and Nanotechnologies, Instituto Superior Técnico, Universidade Técnica de Lisboa, Lisboa, Portugal

*Correspondence:
Luis M. S. Loura, Faculdade de Farmácia, Universidade de Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal.
e-mail: loura@ff.uc.pt;
Manuel Prieto, Centro de Química Física Molecular, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal.
e-mail: manuel.prieto@ist.utl.pt

INTRODUCTION
Förster resonance energy transfer (FRET), in most applications used as a “spectroscopic ruler,” allows an easy determination of the donor-acceptor intermolecular distance. However, the situation becomes complex in membranes, since around each donor there is an ensemble of acceptors at non-correlated distances. In this review, state-of-the-art methodologies for this situation are presented, usually involving time-resolved data and model fitting. This powerful approach can be used to study the occurrence of phase separation (“rafts” or other type of domains), allowing their detection as well as size evaluation. Formalisms for studying lipid-protein and protein-protein interactions according to specific topologies are also addressed. The advantages and added complexity of a specific type of FRET (energy homotransfer or energy migration) are described, as well as applications of FRET under the microscope.

Keywords: energy transfer, fluorescence, lipid bilayers, lipid-protein interaction, lipid rafts

PHENOMENOLOGICAL APPLICATIONS OF FRET IN MEMBRANES
Even if FRET is used as a qualitative indicator of chromophore proximity, without accounting for its actual kinetics, there is still a wide range of applications in membrane biophysics. Additionally, the complexity of some systems is a serious deterrent to the application of complex formalisms, and a more phenomenological approach may be the only option available.

A classic use of FRET is monitoring lipid exchange or mixing and membrane fusion, such as described by Struck et al. (1981). These authors followed the fusion of phosphatidylserine (PS) vesicles induced by calcium ion. Two vesicle populations, one containing both D and A probes and the other containing solely...
unlabeled phospholipid, were mixed. The chosen FRET pair was composed of the labeled phospholipids N-(7-nitro-2,2,3-
benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE, D) and N-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidy
lethanolamine (Rh-PE, A), and since then has become probably
the most commonly used pair in membrane FRET studies. Upon
addition of calcium ion, fusion causes mixing of the labeled and
unlabeled vesicles. Following lipid redistribution by lateral dif-
fusion, the surface concentration of acceptor probes surrounding
each donor is diminished. This results in a reduced extent of donor
quenching by FRET, the energy transfer efficiency decreases and
therefore the donor emission intensity increases. Another possi-
bility is to monitor the increasing of FRET efficiency (decreasing
quenching by FRET, the energy transfer efficiency decreases and
the D–A distance is the same, such as verified in a solution of a
two-chromophore species, is often termed “intramolecular” FRET.

In the study of lipid lateral distribution, FRET between probes
of identical partition properties will become more efficient as
a consequence of phase separation (and conversely for probes
exhibiting complementary phase preference). However, probe par-
tition is not the only phenomenon that can affect FRET efficiency.

FIGURE 1 | Schematic representation of applications of FRET in
membrane biophysics. Only one bilayer leaflet is depicted. (A) membrane
heterogeneity; (B) determination of transverse location of a fluorescent
residue/label; (C) protein/lipid selectivity; (D) protein oligomerization.

As mentioned below, partition of D or A can be estimated quan-
titatively from the parameters of the time-resolved fluorescence of
D in presence of A (analyzed globally together with that in absence of A). FRET can also be used in a simpler way as an
indicator of domain preference as exemplified by the recently
proposed “FRET assay of raft association” (Nelson et al., 2010).

The efficiency of FRET between the protein under study (D) and one of two acceptors, pyrene–DOPE (prefer ld phase) and
LcTMADPH (prefers lo phase) is measured and compared with
that using other donors, LW peptide (a transmembrane helix
type peptide with high affinity for ld domains) and chola
toxin-B (a protein that binds to the raft-associating lipid gan
glioside GM1 and has a very high affinity for lo domains) in
a ld/lo phase coexistence lipid mixture. In these authors’ exam-
oples, perfringolysin O is thus shown to have intermediate raft
affinity between that of LW peptide and cholera toxin-B in ves-
icles containing ordered domains rich in brain sphingomyelin or
1,2-distearoyl-sn-3-glycerophosphocholine (DSPC).

In the study of lipid/protein interaction, FRET between, e.g., a
membrane peptide or protein D and lipid acceptors of different
classes/acyl chains may indicate preferential association or selectiv-
ity for a particular type of lipid, and FRET between D-bearing and
A-bearing membrane proteins can be used to detect formation of
protein hetero- or homo-oligomers. In this case, quantitative mod-
els are required for further characterization of these interactions
(see Formalisms for lipid–protein or protein–protein interaction
below).

INTRAMOLECULAR FRET: A “SPECTROSCOPIC RULER”

Förster resonance energy transfer between D/A pairs for which
the D–A distance is the same, such as verified in a solution of a
two-chromophore species, is often termed “intramolecular” FRET.
A quantification of the extent of FRET is given by the FRET
efficiency, E, which is calculated as

\[
E = 1 - \frac{\int_0^\infty i_{DA}(t) \, dt}{\int_0^\infty i_D(t) \, dt}
\]

in this equation, \(i_D(t)\) and \(i_{DA}(t)\) are the D decays in absence and
presence of A (respectively). The effect of FRET on the fluores-
cence of D is the reduction of its lifetime and quantum yield. In
this simple case, the D decay law remains exponential, albeit faster
than in the absence of acceptor. The relationship between the life-
time of D in absence and presence of A (\(\tau_0\) and \(\tau\), respectively) is
given by

\[
E = 1 - \frac{\tau}{\tau_0} = \frac{R^6}{(R^6 + R_0^6)}
\]

where \(R\) is the D–A separation. An expression identical to Eq.
2 can be written for the fluorescence quantum yield, or the
Table 1 | Selected examples of FRET membrane studies.

| Application | Reference(s) | Comments |
|-------------|--------------|----------|
| Detection and characterization of membrane heterogeneity | Fung and Stryer (1978) | Pioneering test of Förster theory in fluid egg PC vesicles |
| | Loura et al. (1996, 2000b) | Verification of FRET theoretical decay law in fluid DPPC, but not in the gel, due to probe segregation |
| | Loura et al. (2000a) | Gel/fluid partition of carbocyanine dyes from time-resolved FRET parameters |
| | de Almeida et al. (2001) | Detection of small (~R₀) domains of 10 phase in DMPC/cholesterol |
| | Fernandes et al. (2003) | Kinetics of gel/fluid phase separation in DLPC/DSPC binary mixture |
| | Silvius (2003) | Formation of domains enriched in M13 major coat protein and matching lipid (DOPC) in fluid DEuPC/DOPC and DMoPC/DOPC mixtures |
| | de Almeida et al. (2005) | Dependence of domain size with composition in the ternary POPC/SM/cholesterol system |
| | Basu et al. (2010) | Absence of clustering of PI(4,5)P₂ in POPC at slightly above physiological pH |
| | Beck et al. (2007) | Application of the SP-FRET method to the DOPC/DPPC/cholesterol system |
| | Corne et al. (2009) | Lysozyme induces “pinched lamellar” multibilayer aggregates in POPC:POPS 4:1 mixture |
| Membrane protein mapping | Cha et al. (1999) | Measurement of voltage-sensitive distances between Shaker potassium channel subunits at specific residues |
| | Corne et al. (2009) | Distance measurements in calmodulin bound to the RyR1 Ca²⁺ release channel |
| | Corne et al. (2010) | Distance measurements in the ryamidine receptor FKS06-binding protein subunit |
| | Basu et al. (2010) | Distance measurements in the plasma membrane Cr³⁺ / HCO₃⁻ exchanger, AE1 |
| | Shaklai et al. (1977) | Derivation of Eq. 7 and its application to the minimum distance between hemoglobin heme groups and 12-(9-anthroyl)stearic acid in red blood cell membranes |
| | Gutierrez-Merino et al. (1987) | Derivation of approximate formalism and its application to determine the transverse location of the ATP binding site on the (Ca²⁺ + Mg²⁺)-ATPase |
| | Johnson and Nuss (1994) | Determination of the transverse location of the histricotoxin-sensitive ethidium binding site of the AChR |
| | Valenzuela et al. (1994) | Determination of the transverse location of the agonist binding site of AChR |
| | Yegneswaran et al. (1997) | Determination of the location of the active site of membrane-bound activated protein C relative to the phospholipid surface |
| | Chen and Lentz (1997) | Determination of the location of the heme group of cytochrome c relative to the membrane surface, for varying protein coverage |
| | Domanov et al. (2005) | Determination of the transverse location of the histricotoxin-sensitive ethidium binding site of the AChR |
| | Poveda et al. (2002) | Determination of the location of the active site of membrane-bound activated protein C relative to the phospholipid surface |
| | Gambhir et al. (2004) | Determination of the location of the active site of membrane-bound activated protein C relative to the phospholipid surface |
| | Capeta et al. (2006) | Determination of the location of the heme group of cytochrome c relative to the membrane surface, for varying protein coverage |
| | Poveda et al. (2002) | Verification (using the Gutierrez-Merino formalism) of formation of specific phosphatidic acid-rich lipid domains, caused by AChR, which include the protein |
| | Fernandes et al. (2004) | Derivation of model for single transmembrane α-helix and application to selectivity of M13 major coat protein for different lipids |
| | Gambhir et al. (2004) | PIP2 sequestration by the basic effector domain of myristoylated alanine-rich C kinase substrate |
| | Capeta et al. (2006) | Numerical solutions to lipid-protein selectivity and application to analysis of the data of (Poveda et al., 2002) |
| | Nomikos et al. (2007) | PIP2 sequestration by a basic peptide from phospholipase C-δ |
| | Picás et al. (2010) | Adaptation of the model of (Fernandes et al., 2004) to larger membrane proteins and application to lactose permease lipid selectivity |

(Continued)
### Table 1 (Continued)

| Application | Reference(s) | Comments |
|-------------|--------------|----------|
| Protein–protein oligomerization | Mercier et al. (2002) | β1- and β2-adrenergic receptor homo- and heterodimerization by BRET |
| | Fernandes et al. (2008) | Antiparallel dimerization of the N-BAR N-terminal domain in POPG |
| | Hankumari et al. (2008) | Homodimerization of G protein-coupled secretin receptor by BRET |
| | Fung et al. (2009) | Ligand-regulated oligomerization of β2-adrenoceptors |
| | Harding et al. (2009) | Constitutive dimerization of the G protein-coupled receptor, neurotensin receptor |
| Applications of diffusion-enhanced FRET | Thomas et al. (1978) | Verification of the Steinberg and Katchalski theory in vesicles, using a Tb3+ chelate as donor |
| | Thomas and Stryer (1982) | Determination of transverse location of the retinal chromophore of rhodopsin in membrane vesicles made from disk membranes, using a Tb3+ chelate as donor |
| | Leder et al. (1989) | Determination of transverse location of the retinal chromophore in the purple membrane, using a Tb3+ chelate as donor |
| | Kusba et al. (2002) | Determination of lipid diffusion coefficients using diffusion-enhanced FRET from a Re-chelate-PE to Texas-red PE |
| | Meltzer et al. (2006) | Determination of electrostatic potential at fixed sites on the AChr using diffusion-enhanced FRET, with a Tb3+ chelate as donor |
| FRET microscopy studies | Kenworthy and Edidin (1999) | Clustering of the GPI-anchored protein 5’ nucleotidase was not detected using FRET between labeled antibodies |
| | Varma and Mayor (1998) | Clustering in domains with less than 70 nm of a GPI-anchored protein at the cell surface detected by homo-FRET |
| | Herreros et al. (2001) | FRET-FLIM study of the raft dependent interaction of tetanus neurotoxin with Thy-1 |
| | Hughes et al. (2002) | Suggestion of preferential interaction of phospholipase D with PC, rather than PE – a qualitative FLIM study |
| | Sharma et al. (2004) | Characterization of size of lipid-dependent organization of GPI-anchored proteins in live cells, using homo and hetero-FRET |
| | Von Arnim et al. (2005) | FRET-FLIM revealed interaction between BACE (β site of amyloid precursor protein-cleaving enzyme) and the LDL receptor-related protein occurring on lipid rafts at the cell surface |
| | Acasandrei et al. (2006) | Improved model for analysis of FRET adapted to the case where D and A label two probing proteins. Application to the data of (Kenworthy and Edidin, 1999) gave quantitative support to the presence of lipid rafts |
| | Meyer et al. (2006) | Quantitative study of the distribution of functional neurokinin-1 receptors in the plasma membrane. The receptors are found to be monomeric and reside in membrane microdomains of size below optical resolution |
| | Anikovsky et al. (2008) | Derivation of a model considering intramolecular and/or intermolecular FRET and oligomerization, and its experimental verification. Discussion of the effect of cell fixation |
| | Goswami et al. (2008) | Cortical actin activity regulates spatial organization of nanoclusters of GPI-anchored proteins at the cell surface, as shown by homo-FRET |
| | Hofman et al. (2008) | FRET-FLIM revealed that ganglioside GM1 co-localizes with EGF receptor, but not with the non-raft transferrin receptor |

AChR, nicotinic acetylcholine receptor; DEuPC, 1,2-dieuocoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DMoPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPC, 1,2-oleoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOPS, 1-palmitoyl-2-sphingosyl-1-phosphoethanolamine; DPPS, 1,2-dipalmitoyl-sn-glycero-3-phosphoserine; DSPC, 1,2-distearyloxy-sn-glycero-3-phosphocholine; GPL, glycosylphosphatidylinositol; PC, Phosphatidylincholine; PE, phosphatidylethanolamine; P(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PIP₂, phosphatidylinositol-(4,5)-bisphosphate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol); POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; SM, sphingomyelin; SOPC, 1-stearoyl,2-oleoyl-sn-glycero-3-phosphocholine.  

Fluorescence steady-state intensity, \( R_0 \) is calculated independently from spectroscopic data,

\[
R_0 \quad \text{(in nm units)} = 0.02108 \left[ \kappa^2 \cdot \Phi_D \cdot n^{-4} \cdot \int_0^\infty I(\lambda) \cdot s(\lambda) \cdot \lambda^4 d\lambda \right]^{1/6}
\]

where \( \kappa^2 \) is the orientation factor (see Van Der Meer et al., 1994 for a detailed discussion), \( \Phi_D \) is the D quantum yield in the absence of A, \( n \) is the refractive index, \( \lambda \) is the wavelength (in nm units), \( I(\lambda) \) is the normalized D emission spectrum, and \( s(\lambda) \) is the A molar absorption spectrum. In this way, \( R \) is easily computed from both steady-state and time-resolved data. This is the basis of the use of intramolecular FRET as a “spectroscopic ruler” (Stryer, 1978).

In the context of membrane biophysics, intramolecular FRET is still used to retrieve structural and dynamical information on membrane proteins. This is especially important given that high-resolution structures of many membrane proteins (traditionally obtained using X-ray crystallography, cryo-electron microscopy, or NMR spectroscopy) are still missing. Site-directed labeling allows incorporation of suitable FRET donor and acceptor groups,

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and, from the measurement of FRET efficiency, protein mapping, and/or kinetics of conformational changes can be studied. Recent applications are listed and briefly described in Table 1.

UNIFORM DISTRIBUTION OF FLUOROPHORES IN MEMBRANES

In membranes, each D molecule is usually surrounded by a distribution of A molecules. Therefore, measurement of single D/A distances is neither meaningful nor feasible. The decay of D’s emission becomes complex and dependent on the topology of the system under study, as well as the concentration of A. Analytical solutions can still be derived for uniform distribution of chromophores. For planar distributions of D and A, the decay of D in presence of A is given by Fung and Stryer (1978), Wolber and Hudson (1979):

\[ i_{DA}(t) = \exp\left(-\frac{t}{\tau_0}\right) \exp\left\{-\pi R_0^2 n \left[\frac{2}{3} \left(\frac{R_0}{R_c}\right)^6 \left(\frac{t}{\tau_0}\right)\right]^{1/3}\right\} \times \exp\left\{\pi R_0^2 n \left(1 - \exp\left[-\left(\frac{R_0}{R_c}\right)^6 \left(\frac{t}{\tau_0}\right)\right]\right)\right\} \]

In this equation, \(\gamma\) is the incomplete gamma function, \(R_c\) is the minimum D/A distance (exclusion distance), and \(n\) is the numerical concentration of A (molecules/unit area). Although it was originally derived for a plane of acceptors containing the donor (cis transfer), it is also valid if the D molecule is separated from the A plane by a distance \(R_c\), a situation common on membranes, as D and A are often located at different depths in the bilayer.

Upon preparation of lipid vesicles, D and A molecules are frequently inserted in either of the bilayer leaflets, with equal probability. In this case, one must consider two planes of A for a given D, one corresponding to the acceptors lying in the same bilayer leaflet as the donor, and another for those located in the opposite leaflet. The decay law in this case is obtained by simply multiplying the intrinsic D decay by the FRET terms corresponding to each plane of A.

Another common occurrence in membrane systems is a complex decay of D even in the absence of A, with a sum of two or three exponentials being required for a proper description. In this case, the above equations can be still used, provided that the exponential D intrinsic decay term is replaced by this function, and \(\tau_0\) is replaced by the intensity–average (Lakowicz, 2006) decay lifetime. This is so because each lifetime component is characterized by a different \(R_0\) value, proportional to the inverse sixth power of the respective fluorescence quantum yield \(\Phi_D\) (Eq. 3). If all these components have identical radiative decay constants (usually a good approximation), their \(\Phi_D\) values are in turn proportional to those of \(\tau_0\). This implies that the FRET rates to a given A located at distance \(R\) given by \((1/\tau_0)(R_0/R)^6\) are the same irrespective of the D lifetime component considered, because \(R_0^6/\tau_0\) is invariant (Loura et al., 1996, 2000b). Because of this constancy, Eq. 4 can be used with the average values of \(R_0\) and \(\tau_0\). The option here is to use the spectroscopic \(R_0\) (calculated with the experimental, averaged value of \(\Phi_D\) and the true statistical average of \(\tau_0\), which is the intensity–average.

For steady-state applications, Eq. 4 can be integrated numerically (in a program or spreadsheet) to produce curves of FRET efficiency \(E\) (calculated using Eq. 1) as function of acceptor concentration \(n\) with \(R_c\) as a parameter. Alternatively, \(R_c\) is fixed and experimental FRET decays/efficiencies are compared with theoretical expectations. Eventual failure to analyze FRET kinetics with the uniform probe distribution formalism may have relevance (e.g., addition of a new component to a given one-phase lipid bilayer system may induce compartmentalization and/or phase separation).
can produce insights regarding the size of the domains, and in the case of infinite phase separation (which can be validated if the “FRET” and “non-FRET” $K_{PA}$ values are indistinguishable) it was shown that the phase diagram of a binary mixture can be obtained from the decay parameters (Loura et al., 2001). An experimental method of characterization of phase separation in lipid membranes (and determination of binary and ternary phase diagrams in the infinite phase limit) based on this formalism, but relying solely on acceptor steady-state sensitized emission, was proposed by Buboltz (2007), who termed it “Steady-State Probe-Partitioning FRET” or SP-FRET. Applications of these methodologies are listed in Table 1.

**NUMERICAL AND SIMPLIFIED ANALYTICAL TREATMENTS OF FRET IN NON-HOMOGENEOUS SYSTEMS**

No exact solution of the FRET rate or efficiency has been derived for the case of incomplete phase separation, with nanometer-sized domains of a given type dispersed in the continuous phase. This stems from the evident symmetry loss introduced by the presence of the domains. One way to tackle this complexity is to calculate the D decay using numerical simulation. Basically, the process starts by building a topology of the lipid matrix (i.e., define size of the simulated system, domain shape, average size and size distribution, and then place the domains on the matrix, ensuring that the overall fraction of each phase is as intended) and placing D and A molecules taking into account their domain preference. A given D is then selected and its interaction with all the acceptors (or those within a cutoff of several $R_0$ lengths) is computed. This step is then repeated for all the donors in order to obtain an ensemble average value for all the system. One obtains the average D decay, and, by using Eq. 1, the FRET efficiency value.

This kind of numerical simulations was already described by Wolber and Hudson (1979) for uniform distribution in a planar geometry to test their analytical theory. The first numerical solution of FRET for non-uniform membrane probe distribution was given by Snyder and Freire (1982). In this work, heterogeneity of probe distribution was introduced by incorporating a heuristic potential function in the random placement of the probes. Therefore, no domains are actually simulated, and whereas the authors’ equations are suited to the analysis of probe aggregation in a single phase system, they are not useful regarding phase separation. Simulations in which probe distribution heterogeneity is introduced by building a biphase system and taking into consideration probe partition have been presented by authors to provide tests for their analytical formalisms (Loura and Prieto, 2000; Loura et al., 2001; Towles and Dan, 2007; Towles et al., 2007).

Another simulation approach consists in recreating the excitation and de-excitation processes of each D or A molecule by performing stochastic simulations. These calculations take into account the probabilities of donor excitation, donor decay by non-FRET processes, FRET to a given acceptor, and acceptor de-excitation. The FRET efficiency is simply calculated by the ratio between the total number of transfers and the total number of D excitations. This type of simulation has been applied to the case of FRET in a planar geometry with dislikle domains (Kiskowski and Kenworthy, 2007).

All previous works are characterized by prior fixing of the underlying lipid matrix, including domain size and shape, and the simulations concern exclusively the calculation of FRET rates and probabilities. A different approach was recently undertaken by Frazier et al. (2007), who combined statistical mechanical lattice Monte-Carlo simulations (to describe the lipid matrix and to generate chromophore positions therein) with a simplified step-function FRET distance dependence (FRET was considered to occur if and only if the D–A distance in a given pair were less than $R_0$) to analyze experimental data of FRET in a ternary, raft-model mixture. This work demonstrates that FRET and computational techniques can be combined to create a powerful combination, suited to the study of lipid phase separation.

Globally, numerical simulations have the advantage of allowing the calculation of FRET in systems for which, due to their complexity, an exact solution is precluded. However, thus far they do not provide a way to analyze experimental data directly, as many degrees of freedom are expected in an actual experiment. For each simulation, values for $R_0$, D lifetime, D/A exclusion distance (all being possibly different in each coexisting phase), domain shape and size, and D and A partition coefficients need to be fixed. This multiplicity of variables excludes the possibility of fitting with simple empirical functions that could describe, e.g., the variation of FRET efficiency as a function of concentration of A in a general manner, which would be convenient for most researchers. The study of a particular system requires individual setup and simulation procedures.

An alternative to numerical simulations of FRET in nanoheterogeneous bilayers is the use of simplified analytical treatments, which, unlike the former, could potentially be suited to direct analysis of experimental data enabling recovery of the parameters of interest. However, this kind of formalisms has been characterized by either severe simplifying approximations (Gutierrez-Merino, 1981; Brown et al., 2007a,b) or relying to some extent to numerical results (Towles et al., 2007; see Loura et al., 2010a for a detailed discussion), precluding their widespread use.

**FORMALISMS FOR LIPID–PROTEIN OR PROTEIN–PROTEIN INTERACTION**

A relatively simple application of FRET in membrane systems containing peptides/proteins bearing fluorescent residues (tryptophan and tyrosine) is the determination of the transverse location of the latter. Tryptophan and tyrosine act as energy transfer donors, since they absorb at short wavelengths, so suitable acceptors with known positions in the membrane should be used (see Table 1). Because the efficiency of transfer depends on the D–A exclusion distance, the value of $R_0$ (and hence the transverse location) can be obtained by fitting Eqs 1 and 4 to the experimental data. A series of stearic acids derivatized with the anthroyl chromophore is available, and is characterized in great detail in the literature (see e.g., Blatt et al., 1984). Because there is almost invariance of the absorption spectra of the different acceptors, the Förster radius is constant for all of them, and for tryptophan as D it is close to $R_0 = 25 \text{ Å}$. It can be shown that, when $R_c > \text{approximately 1.7 } R_0$, a Stern–Volmer type dependence is obtained (Shaklai et al., 1977; Dewey and Hammes, 1980), expressed by the very simple equation

$$K_{PA} = \frac{R_0^6}{8\pi^2 \epsilon_0 c \mu_n^2}$$
that of combined inter-aggregate and intermolecular FRET (You et al., 2005). If the donor population is subjected to quenching by two different types of acceptor (e.g., those bound and unbound to a given donor), the correct way to calculate the FRET efficiency is to multiply the FRET terms corresponding to all quenching contributions to obtain $I_{DA}(t)$, and integrate in the end (Eq. 1). This is exemplified in a recent study, which concluded that the N-BAR N-terminal domain forms antiparallel dimers in 1-palmitoyl-2-oleoyl-sn-glycerol-3-[phospho-rac-(1-glycerol)] (POPG) vesicles (Fernandes et al., 2008).

**DIFFUSION-ENHANCED FRET**

In the preceding sections, it was assumed that translational diffusion of both D and A were negligible during the lifetime of the D excited state, that is, the condition $D_{DA} \tau_0 / s^2 \ll 1$ (where $D_{DA}$ is the sum of D and A lateral diffusion coefficients and $s$ is the average D–A distance) was met. Otherwise, fast chromophore diffusion leads to an enhancement in the rate of FRET, which can be intuitively understood by considering that a D/A pair, initially separated by a large distance which would prevent FRET, might become close enough for FRET to occur with high probability in the course of the D excited state lifetime as a consequence of diffusion.

The general theory of diffusion-enhanced FRET was established by Steinberg and Katchalski (1968) and verified experimentally by Thomas et al. (1978). Of particular interest is the so-called rapid-diffusion limit, characterized by $D_{DA} \tau_0 / s^2 \ll 1$, which leads to extremely simplified equations for the rate of FRET. This condition requires D lifetimes in the microsecond to millisecond range, which are typically achievable using suitable lanthanide chelates as the D species. For a uniform two-dimensional distribution of uncharged spherical chromophores, it can be shown that an exponential D decay is obtained, with lifetime

$$\tau = \frac{1}{(\tau_0^{-1} + k_T)}$$

where

$$k_T = \frac{\pi n R_0^4}{2 \tau_0 R_e^4}$$

The latter equation (which, rather curiously, is formally identical to Eq. 7, which is an approximate solution to a very different problem) shows that the rate of FRET in this regime is highly dependent on $R_e$. This is the reason why rapid-diffusion limit FRET found application in the measurement of the distance of closest D–A approach (where typically the A species is a protein), in the 1980s (see Table 1 for examples). Additionally, FRET-enhanced diffusion of charged D/A species is sensitive to the electrostatic potential, and therefore can be used to determine membrane potentials (Meltzer et al., 2006). Surprisingly, recent applications in membranes have been very few, which led to the spirited comment “Since the mid 1980s, the technique [diffusion-enhanced FRET] seems to have remained dormant waiting for the right opportunity to spring back to life” (Fairclough, 2006).

**HOMO-FRET vs. HETERO-FRET**

In the preceding sections, it was assumed that the D and A chromophores were distinct (hetero-FRET). This implied the irreversibility of the transfer process, which could be monitored measuring either the extent of quenching of D or the sensitized fluorescence of A. In contrast, FRET between identical fluorophores (homo-FRET) does not lead to a reduction in donor fluorescence intensity or lifetime, because the donor excited state population is not diminished during the act of transfer. In practice, the
sole observable which reflects the phenomenon is fluorescence anisotropy (Lakowicz, 2006), which is reduced as a consequence of homotransfer. Measurement of fluorescence anisotropy requires polarizers and, because these lead to a considerable reduction in the detected emission, often a larger amount of fluorophore (relative to that which would be used in an intensity measurement) is needed for a given precision. In case that instrumentation is not a problem, the decrease in anisotropy is quite clear: if the two molecules are separated by distance \( R = R_0 \), the measured anisotropy will only be \( \sim 2/3 \) of that of the monomer, which is a significant decrease.

Despite having an obvious advantage of only requiring a single fluorophore, the use of homo-FRET is more restricted than that of hetero-FRET. The rationalization of the extent of depolarization due to homo-FRET is more complicated than that of quenching due to hetero-FRET, because: (i) there is the possibility of back-transfer to the directly excited donor, or transfer to any donor, eventually involving a large number of transfer steps, and (ii) being fluorescence anisotropy the relevant observable, in addition to RET, another source of depolarization is fluorophore rotation. If rotation and RET occur in the same timescale, the two phenomena are coupled, which constitutes the main obstacle to quantitative data analysis of homotransfer. Theoretical descriptions which do not take these features into proper account (e.g., Yeow and Clayton, 2007) should be viewed cautiously. Despite this complexity, homo-FRET is still regularly used in membrane studies, more recently in combination with fluorescence microscopy (see section below and Table 1; Bader et al., 2011) to detect and characterize chromophore (e.g., labeled protein) confinement or aggregation. In this regard, it should be noted that the use of high numerical aperture objectives results in reduction of the observed anisotropy (Axelrod, 1979). This artifact can be minimized using a lower numerical aperture (≤0.8), with loss in resolution and sensitivity, or corrected, e.g., by using a standard molecule (Tramier and Coppey-Moisan, 2008).

**FRET UNDER THE MICROSCOP**

Recent developments in multi-wavelength and polarization resolved imaging have led to a widespread use of FRET imaging in studies of functional assemblies in cell membranes. The experimental methods for visualizing membrane microdomains and quantifying FRET efficiencies in FRET microscopy with emphasis on novel strategies have been reviewed elsewhere (Rao and Mayor, 2005; Jares-Erijman and Jovin, 2006; Owen et al., 2007; Padilla-Parra et al., 2008). Several approaches were developed in order to explore, on the nanoscale range, specific protein–protein, lipid–lipid, or lipid–protein interactions in live cells, both using homo- and hetero-FRET.

Cell membranes are characterized by a large number of lipid and protein components in a non-equilibrium state. One common simplification is to assume two types of domains, e.g., raft/non-raft or ordered/disordered. The results can then be compared to, e.g., the Id/lo coexistence on a lipid phase diagram in a ternary model system. Due to intrinsic limitations such as cell stability, and because usually in cells microscopy studies are carried out (in order to control cell state, to know the fluorophore localization, and use the signal coming only from the membrane of interest) fluorescence intensity decays with a high number of photons and low background signal (necessary to the applications of most of the formalisms described above) are generally unfeasible. Usually, steady-state data is obtained and compared to an integrated FRET formalism. Even when fluorescence lifetime imaging microscopy (FLIM; see Stöckl and Herrmann, 2010) for a review of its applications to membrane heterogeneity) lifetime data is obtained (FRET–FLIM), a relatively low number of counts is often obtained, which implies that the decay is traditionally used to calculate FRET efficiency using Eq. 1, rather than directly analyzed with the underlying FRET kinetic model. However, with instrumental improvements as well as development of novel analysis approaches (Grecco et al., 2009) this trend is being reversed. Selected works combining FRET and microscopy are listed in Table 1, which succinctly describes illustrative literature reports in which FRET was used in (at least) one of the applications described above.

**CONCLUSION**

In this review, applications of FRET in membrane biophysics are described, comprising studies of membrane protein mapping, lateral heterogeneity (membrane domains), determination of the transverse location (depth) of fluorescent residues/labels inside the membrane, protein/lipid selectivity (preference of a specific lipid for the protein vicinity), and membrane protein oligomerization.

The complexity of FRET in membranes was addressed, an evaluation of hetero vs. homo-FRET is presented, and detailed topological information can be obtained from this methodology, once adequate modeling is taken into account. Examples of relevant works in this area are critically reviewed, and the recent applications of FRET under the microscope, namely from time-resolved data (FRET–FLIM), are mentioned. On the whole, the power of FRET as a tool in membrane biophysics is emphasized.

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