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A SIMPLE “PROXIMITY” CORRECTION FOR FRET EFFICIENCY DETERMINATION IN MEMBRANES USING LIFETIME MEASUREMENTS

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Abstract

Accurate measurements of oligomerization in membranes by FRET are always compromised by a substantial contribution from random chance colocalization of donors and acceptors. Recently some of us [*Biochemistry* 2005, 44:352–360] demonstrated the use of computer simulation in estimating the contribution of this “proximity” component to correct the FRET efficiency and estimate the free energy of dimer formation of the G380R mutants of fibroblast growth factor receptor 3 (FGFR3) transmembrane domain immersed into lipid bilayer. Because tight dimerization will result in complete energy transfer from donor to acceptor, we have used the same experimental system of Fluorescein- and Rhodamine-labeled G380R mutants of FGFR3 for the experimental assessment of the “proximity” FRET corrections using fluorescence lifetime measurements. The experimental “proximity” FRET correction, based on time-resolved fluorescence measurements, is expected to have general advantages over theoretical correction, especially in case of non-randomly distributed monomers.

Keywords

lipid bilayer; dimerization; Förster resonance energy transfer; static fluorescence quenching; dynamic fluorescence quenching; fluorescence lifetime

In a typical bilayer FRET experiment, the observed transfer efficiency will always contain contributions from specific dimerization and from random colocalization (i.e. when FRET is due to random proximity of the acceptors and donors), (see Fig.1). To obtain FRET efficiency that is due only to dimerization, one must subtract FRET efficiency that is due to proximity from the FRET signal measured by steady state fluorescence spectroscopy. One of the possible ways to estimate the contribution from this “proximity” component is computer simulation of FRET for randomly distributed fluorophores. In this calculation, carried out first by Wolber and Hudson (1) and simulated by Wimley and White (2) and by Li and co-workers (3), FRET from randomly distributed peptides is determined by averaging the donor

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quenching by acceptors in a specific configuration over a large number of acceptor configurations. The efficiency of FRET (E) of a donor by a specific acceptor configuration is given by

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

where r_i is the distance between the donor and the i th acceptor in the system and R_0 is the Förster radius for the donor/acceptor pair. One of the potential problems of this modeling approach, however, is the limitation imposed by the need to assume the random distribution of fluorophores on the plane of the bilayer.

Here we present an alternative way to experimentally account for the “proximity-FRET”, which is based on a comparison of steady-state and time-resolved fluorescence measurements, and does not require or assume a random distribution of fluorophores. Because tight dimerization results in a complete energy transfer from donor to acceptor the quenching of the donor will be static, while donor quenching due to random proximity will be dynamic. Time-resolved measurements can therefore be used to distinguish between dimerization and random proximity. We have used Fluorescein- and Rhodamine-labeled G380R mutants of FGFR3 TM domain (*i.e.*, the same experimental system as in (4, 5)) to test this hypothesis. The LUV (POPC) samples contained fluorescein- (donor) and rhodamine-labeled (acceptor) FGFR3 peptides together as well as control LUV (POPC) samples containing only fluorescein-labeled and only rhodamine-labeled FGFR3 peptides. Samples were prepared as described in ref. (3).

Steady-state fluorescence was measured using an SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. Excitation wavelength was set at 439 nm. Excitation slits were 1 nm; emission slits were 4 nm. Fluorescence emission spectra were obtained by averaging 5–10 scans collected over a 450–750 nm range using 1 nm steps. The measurements were made in 4×10 mm cuvettes, oriented perpendicular to the excitation beam and maintained at 25°C using a Peltier device from Quantum Northwest (Spokane, WA) with a 0.1°C accuracy.

Energy transfer total efficiency, E_{ss} , was calculated from steady state fluorescence measurements of donor intensity at 519 nm (for Fl/Rh) in the absence and presence of the acceptor.

$$E_{\text{ss}} = \frac{I_{\text{D}} - I_{\text{DA}}}{I_{\text{D}}} \quad (2)$$

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

Fluorescence decays were measured with a time-resolved fluorescence spectrometer FluoTime 200 (PicoQuant, Berlin, Germany) using a standard time-correlated single-photon counting scheme as described previously (6). Samples were excited at 439 nm by subnanosecond pulsed diode laser (LDH 440, PicoQuant, Berlin, Germany) with a repetition rate of 10 MHz. Fluorescence emission of the donor dye was detected at 519 nm via a

polarizer set at 54.7°. The fluorescence intensity was recorded within 4096 channels (34 ps/channel). Data were normally collected to a constant peak value of ten thousand counts. The instrumental response function (IRF) was recorded under the same conditions at the excitation wavelength by replacing the sample with a scattering solution of colloidal silica (LUDOX, Grace).

The fluorescence intensity decay was analyzed using FluoFit (PicoQuant) program. The program uses an iterative fitting procedure based on the Marquardt algorithm to minimize the deviation of the experimental data, presented as the sum of the exponential components:

$$I(t) = \sum_i \alpha_i e^{(-t/\tau_i)} \quad (3)$$

where α_i are the pre-exponential amplitudes and τ_i are the lifetimes of the decay components. Normally, a tri-exponential decay was used in which a lifetime of the shortest component was fixed at 0.3 ns. This component, originating mostly from membrane scattering (6), contributes negligibly into total intensity and was ignored in a subsequent analysis. The precision of decay parameters was analyzed using support-plane analysis (7). The nature of the deviation of the decay from a mono-exponential function is not understood in this system (as in many other cases), with both conformational heterogeneity and excited state reaction being possible contributors. As an approximation, we have characterized time-resolved FRET using an amplitude-average lifetime, calculated as follows:

$$\tau_\alpha = \frac{\sum_{i=1}^n \alpha_i \tau_i}{\sum_{i=1}^n \alpha_i} \quad (4)$$

FRET efficiency for randomly distributed G380R-FGFR3 peptides labeled with Fluorescein and Rhodamine (E_{TR}) was calculated from the amplitude-average lifetime of donor in the absence and presence of the acceptor:

$$E_{TR} = \frac{\tau_\alpha^D - \tau_\alpha^{DA}}{\tau_\alpha^D} \quad (5)$$

where τ_α^D and τ_α^{DA} are the amplitude-average lifetimes of the donor decay at 519 nm in the absence and presence of the acceptor, correspondingly.

According to the results of steady-state and time-resolved measurements presented in Table 1, the addition of the acceptor-labeled peptide results in decrease of steady-state relative fluorescence intensity, in an increase of the contribution of the shortest component of fluorescence decay (+0.04% RRG380R_Rh sample) and in a substantial shortening of both lifetimes (+0.15% G380R_Rh sample).

The increase of the contribution of the shortest fluorescence decay component is likely to be a manifestation of the complex nature of quenching in membranes. Each excited fluorophore can interact with several quenchers that are located at different distances on a membrane and that, therefore, have different quenching efficiencies. The strongly quenched fluorophores will contribute to a short lifetime component, and α_1 will increase as a result. In principle, such quenching could be modeled by a continuous lifetime distribution, but the practical

advantages of doing so are highly questionable for a system in which fluorescence decay is heterogeneous even before quenching (the fluorescence of only donor-labeled peptides can be adequately described by a biexponential decay, see Table 1). To avoid over interpretation of the decay parameters, we concentrated on analyzing the behavior of the amplitude-weighted average lifetimes, τ_w , which are also shortened on the addition of the acceptor-labeled peptide (Table 1).

The time-resolved FRET efficiency (E_{TR}), calculated by Eq.5, was found to be always significantly smaller than obtained in a steady-state experiment (E_{SS}). Assuming that in this case the dimerization results in a purely static quenching, we have corrected E_{SS} for “proximity” contribution, approximated by E_{TR} . This experimentally corrected FRET efficiency ($E_{Exp. Corr.}^{Exp.}$) exactly coincided with that corrected using theoretical considerations ($E_{Theor. Corr.}^{Theor.}$), (Table 1). Such coincidence of theoretically and experimentally corrected FRET efficiencies indicates that the assumption of random distribution of donors and acceptors is valid for the used experimental system of Fluorescein- and Rhodamine-labeled G380R mutants of FGFR3 in LUV (POPC).

The experimental “proximity” FRET correction, based on steady-state and time-resolved measurements described here, is simple to implement and has important advantages over theoretical correction. Most importantly, unlike the theoretical correction, it can be used when probe monomers are not randomly distributed. Examples of non random distributions include bilayers that have phase-separated lipid domains, experimental systems that have a difference in the distance of the dye molecules from the bilayer surface, or systems in which long range electrostatic interactions between molecules alters their distribution in the plane of the bilayer.

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ABBREVIATIONS

FRET	Förster resonance energy transfer
FGFR3	fibroblast growth factor receptor 3
TM	transmembrane
LUV	extruded Large Unilamellar Vesicles of 100 nm diameter
POPC	palmitoylcholinephosphatidylcholine
Fl	fluorescein
Rh	rhodamine
IRF	instrumental response function

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Figure 1.

Schematic illustration of the FRET-based analysis of dimer formation in membranes. “D” and “A” denote donor- and acceptor-labeled molecules capable of dimerization. A₁ corresponds to the acceptor in a donor-containing dimer, which results in complete energy transfer from donor to acceptor and static quenching of the donor (see text); A₂ corresponds to the acceptor attached to a randomly diffusing monomer and is causing dynamic proximity-FRET quenching of the donor; A₃ is attached to a distant monomer ($R > 2R_0$, R_0 - Förster radius, which equals 55 Å for the FI-Rh pair used here) and causing no quenching.

Fluorescence steady state and decay parameters, FRET efficiencies and “proximity” corrections determined from steady state and time-resolved measurements of membranes containing mixtures of RRG380R mutants of FGFR3 peptide labeled with donor (FI) and acceptor (Rh).

Table 1

Sample	Relative Fluor. Intensity		Decay Parameters						E_{SS}	E_{TR}	$E_{Exp}^{Exp, Corr.}$	$I_{E^{Theor, Corr.}}$
	I_D	I_{DA}	τ_1 ns	a_1	τ_2 ns	a_2	τ_a	τ_a				
0.125%RRG380R_FI	1	-	2.9	0.49	4.7	0.51	3.8	-	-	-	-	-
0.04%RRG380R_FI+0.04%RRG380R_Rh	1	0.91	2.9	0.54	4.7	0.46	3.7	0.09	0.03	0.06	0.06	0.06
0.15%RRG380R_FI+0.15%RRG380R_Rh	1	0.75	2.4	0.49	4.3	0.51	3.4	0.25	0.11	0.14	0.14	0.14

I from (4).

Note: I_D and I_{DA} are the steady-state relative donor intensities of samples containing only donor-labeled peptides and samples with both donor- and acceptor-labeled peptides, respectively. The amplitude-averaged lifetimes τ_a are defined by Eq.3. The experimentally corrected FRET efficiency ($E_{Exp}^{Exp, Corr.}$) is calculated as a difference between the total FRET efficiency obtained in a steady-state experiment (E_{SS}) and “proximity” FRET efficiency obtained in a time-resolved experiment (E_{TR}). The relative errors of E_{SS} , E_{TR} and $E_{Exp}^{Exp, Corr.}$ were less than 1%, 15% and 10%, respectively. $E^{Theor, Corr.}$ - theoretically corrected FRET efficiency (4)