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Joint refinement of FRET measurements using spectroscopic and computational tools

Alexander Kyrychenko [a, b, *], Mykola V. Rodnin [b], Chiranjib Ghatak [b], Alexey S. Ladokhin [b, **]

[a] Institute of Chemistry and School of Chemistry, V. N. Karazin Kharkiv National University, 4 Svobody Square, Kharkiv 61022, Ukraine
[b] Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, Kansas City, KS 66160-7421, USA

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ABSTRACT

The variability of the orientation factor is a long-standing challenge in converting FRET efficiency measurements into donor-acceptor distances. We propose the use of molecular dynamics (MD) simulations to characterize orientation distributions and thus improve the accuracy of distance measurements. Here, we test this approach by comparing experimental and simulated FRET efficiencies for a model donor-acceptor pair of enhanced cyan and enhanced yellow FPs connected by a flexible linker. Several spectroscopic techniques were used to characterize FRET in solution. In addition, a series of atomistic MD simulations of a total length of 1.5 μs were carried out to calculate the distances and the orientation factor in the FRET-pair. The resulting MD-based and experimentally measured FRET efficiency histograms coincided with each other, allowing for direct comparison of distance distributions. Despite the fact that the calculated average orientation factor was close to 2/3, the application of the average κ^2 to the entire histogram of FRET efficiencies resulted in a substantial artificial broadening of the calculated distribution of apparent donor-acceptor distances. By combining single pair-FRET measurements with computational tools, we demonstrate that accounting for the donor and acceptor orientation heterogeneity is critical for accurate representation of the donor-acceptor distance distribution from FRET measurements.

1. Introduction

Förster Resonance Energy Transfer (FRET) [1] is a powerful tool for studies of structure and dynamics of biological macromolecules and their complexes [2,3], and its applications to fluorescent proteins (FPs) revolutionized studies of cellular systems [4–7]. Despite the popularity of FRET, the fact that its efficiency depends not only on the distance between the donor and acceptor fluorophores, but their mutual orientation, remains a long standing challenge in FRET applications [2,8–10]. In the case of unrestrained and uncorrelated reorientations of fluorophores, the orientation factor in FRET is usually approximated by the limiting value for the fast isotropic rotation $\kappa^2 = 2/3$. The later assumption has been validated by fluorescence anisotropy measurements for freely diffusing dyes or dyes covalently attached by flexible linkers [9]. In many complex biological systems, however, the isotropic averaging is no longer valid due to steric restrictions and slow motions. In such systems the instantaneous orientation factor $\kappa^2(t)$ can vary in the range of 0–4 [2,11], substantially complicating quantitative FRET measurements [8,9,11]. The uncertainties introduced by the orientation factor are largely the reason that the results of single-molecule FRET (sm-FRET) measurements are often presented as a distribution of sm-FRET efficiencies, and not the distributions of donor-acceptor distances [12]. Previously, molecular dynamics (MD) simulations have been applied in studies of FRET in lipid membranes by providing information on transverse localization and relative orientation of fluorophores [13–15]. Here we suggest using MD simulations for a benchmark donor-linker-acceptor system to estimate FRET orientational factors, which can improve the recalculation of donor-acceptor distance information from sm-FRET measurements.

* Corresponding author. Institute of Chemistry and School of Chemistry, V. N. Karazin Kharkiv National University, 4 Svobody Square, Kharkiv 61022, Ukraine.
** Corresponding author.
E-mail addresses: a.v.kyrychenko@karazin.ua (A. Kyrychenko), aladokhin@kumc.edu (A.S. Ladokhin).

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2. Materials and methods

2.1. Sample preparation

Enhanced cyan (ECFP) and enhanced yellow fluorescent (EYFP) proteins were linked with a flexible Gly/Ser peptide linker composed of 3 repeating units of GlyGlySerGlyGlySerGlyGlySer [16], referred to as l_3 (Fig. 1A). The pET28b plasmid containing ECFP-l_3-EYFP construct was purchased from Addgene company (product code 21 762, Cambridge, MA). For the protein expression, the *E. coli* BL21 (DE3) competent cells were transformed with this plasmid and grown on the LB agar plates containing 50 mkg/mL kanamycin. Single colony was grown overnight in 5 mL of LB medium containing kanamycin and inoculated in 500 mL of LB/Kan medium, grown to OD 0.6. After reaching the mentioned OD cells were induced with 0.1 mM IPTG and grown overnight at 25 °C. In the morning cells were harvested by centrifugation at 5000*g* for 15 min, suspended in lysis buffer 50 mM Tris-HCl, pH 8, 300 mM NaCl and 5 mM imidazole. Then the cells were lysed by sonication for 1 min (lysozyme in concentration 10 mg/mL and Complete protease inhibitor (Roche, Pleasanton, CA) were added prior to sonication) and the soluble fraction was obtained by centrifugation at 15000*g* for 30 min. The soluble fraction was bound overnight with Ni-NTA resin (Qiagen, Germantown, MD), after 4 washes with lysis buffer the target protein was eluted with 0.5 M imidazole in lysis buffer. The ECFP-l_3-EYFP containing fractions were then passed through 1 × 30 cm Superose 12 column in 50 mM phosphate buffer, pH 8 and analyzed by SDS-PAGE, showing >95% the protein purity.

For the preparation of donor alone (ECFP) the stop codon was introduced at the 3′ terminus of the CFP sequence in ECFP-l_3-EYFP construct. For the preparation of acceptor alone (EYFP) the sequence was PCR amplified using ECFP-l_3-EYFP as a template with primers containing NdeI site (GCACCCATATGGTGAGCAAGGGCGAG forward primer) and EcoRI site (CCACGAATTCGGACTTGTACAGCTC reverse primer) and ligated into pET28b plasmid restricted with the same enzymes. Both donor and acceptor fluorescent proteins were expressed and purified under the condition used for ECFP-l_3-EYFP construct, following in general the protocol described in Ref. [16].

2.2. Trypsin limited proteolysis

Trypsin was added to ECFP-l_3-EYFP (10 mkM) at enzyme:substrate ratio 1:1000 and fluorescent spectra were recorded beginning at 0 min in short intervals. The reaction was conducted at room temperature in the 50 mM phosphate buffer at pH 8, which corresponds to the optimal cleavage conditions with trypsin.

2.3. Steady-state and time-resolved fluorescence

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. The measurements were made in a 2 × 10 mm cuvette oriented perpendicular to the excitation beam and maintained at 25 °C using a Peltier device from Quantum Northwest (Spokane, WA). Fluorescence decays were measured with a time-resolved fluorescence spectrometer, FluoTime 200 (PicoQuant, Berlin, Germany), using a standard, time-correlated, single-photon counting scheme [17,18]. Depending on measurement setup, samples were excited at either 373 nm or 440 nm by a subnanosecond pulsed diode laser, LDH 375 or 440, respectively, (PicoQuant, Berlin, Germany), operating with a repetition rate of 10 MHz. Fluorescence emission was selected by a Sciencetech Model 9030 monochromator, using a PMA-182 photomultiplier (PicoQuant, Berlin, Germany) [17]. The fluorescence intensity decay was analyzed using FluoFit iterative-fitting software based on the Marquardt algorithm (PicoQuant, Berlin, Germany). All measurements were carried out in the 50 mM phosphate buffer at pH 8.

2.4. Fluorescence correlation spectroscopy

FCS measurements and analysis were performed as previously described [17]. The samples contained 1 nM of the protein. The measurements were conducted on a MicroTime 200 confocal microscope (PicoQuant, Berlin, Germany), where the sample was excited with a pulsed picosecond diode laser, LDH-P-C-470, λ_{ex} = 470 nm, operated at 40 MHz. To suppress influences from the after-pulsing typically observed with single photon avalanche diodes (SPAD), the fluorescence light was split with a 50/50 beam splitter cube onto two SPADs (SPCM-AQR-14; Perkin-Elmer Inc.), referred to as Donor and Acceptor channel. To distinguish the two fractions of the collected emission, the Donor and Acceptor fluorescence was further optically separated: Before entering into the Donor channel, the emission passed through an emission bandpass filter (AHF/Chroma: HQ 485/55). The emission in the Acceptor channel was acquired after passing through an emission long-pass filter (AHF/Chroma: OG 560). The high numerical aperture apochromatic water immersion objective (60, NA 1.2; Olympus), together with the 50 μm confocal pinhole, resulted in a confocal detection volume of 1 fL. The fluorescence was detected applying time-correlated single-photon counting (TCSPC) with the TimeHarp 200 board. The data were stored in the time-tagged time-resolved mode (TTTR), which allowed the recording of every detected photon with its individual timing and detection channel information.

To estimate single-molecule FRET (sm-FRET) efficiency, the number of fluorescence photons was measured for the Donor (I_D) and for the Acceptor (I_A) channel, as obtained from bursts of photons originating from freely diffusing ECFP-l_3-EYFP. The FRET efficiency E is defined by the ratio of the number of quanta transferred from D to A over the total number of quanta absorbed by D. The related transfer efficiency can be calculated by Eq. (1) [19]:

$$E_{FRET} = \frac{I_A}{I_A + \gamma \cdot I_D} \tag{1}$$

where a correction factor γ takes into account different detection efficiencies of photomultipliers in Donor and Acceptor channels (I_D and I_A), as well as for direct excitation of the acceptor and donor (cross-talk) leakage. For our FCS setup, γ was found to be 2.38.

2.5. FRET analysis

The energy-transfer efficiency E_{obs} was calculated from the decrease in steady-state fluorescence and the fluorescence lifetime of the donor, observed in the presence of the acceptor, according to Eq. (2) [20]:

$$E_{obs} = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D} \tag{2}$$

where F_{DA} (τ_{DA}) and F_D (τ_D) are the fluorescence intensities and lifetimes of ECFP in the presence and the absence of the acceptor, respectively.

ECFP protein exhibits highly complex fluorescence decays showing strong temperature and pH dependencies [21]. The fluorescence intensity decay was analyzed using FluoFit software (PicoQuant, Berlin, Germany). The FluoFit program uses an iterative-fitting procedure based on the Marquardt algorithm to