Biophysical Journal, Volume 97

Supporting Material

Quantitative comparison of different fluorescent protein couples for fast FRET-FLIM acquisition

Sergi Padilla, Nicolas Audugé, Hervé Lalucque, Jean Claude Mevel, Maïté Coppey-Moisan, and Marc Tramier

SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Plasmid constructs

The plasmids coding the mCherry-EGFP or EGFP-EGFP tandems have been described elsewhere (1). The plasmid coding the mStrawberry-EGFP tandem was obtained thanks to the same strategy used for mCherry-EGFP (1). In short, the EGFP fragment from pEGFP-C1 (Clontech, city, USA) is replaced by the mStrawberry coding sequence from pRSET-mStrawberry (generous gift of Dr. R. Tsien, UCSD, USA) using the BsrGI and NheI sites, leading to pmStrawberry-C1. The first EGFP sequence in pBunny (tandem EGFP-EGFP, (1)) is then replaced by the mStrawberry sequence from pmStrawberry-C1 using NheI and BspEI sites, giving the mStrawberry-EGFP plasmid. The mTFP1-mOrange tandem, pmOrange-N1 and pmTFP1-C1 plasmids are generous gifts from Dr O. Albagli (IGR, Villejuif, France). To obtain the mTFP1-EGFP plasmid, the first EGFP sequence in pBunny (tandem EGFP-EGFP, (1)) is substituted by the mTFP1 coding sequence from pmTFP1-C1 using NheI and BspEI sites. The mTFP1-EYFP plasmid is constructed by replacing the EGFP fragment from mTFP1-EGFP plasmid by the EYFP sequence from pEYFP-N1 (Clontech) taking advantage of BamHI and NotI sites. To construct the plasmid coding the Halotag-EGFP tandem using PCR, the sequence containing the linker and EGFP are amplified by PCR from mCherry-EGFP with the primers CTGGCCGGCATGGACGAGCTGTACAAGTCC-3' (the underlined NaeI site is added to facilitate the cloning) and 5'-CTCTACAAATGTGGTATGGC-3'. The PCR product is then digested by NaeI and NotI and cloned into the Halotag pHT2 vector (Promega) hydrolysed by the same enzymes, leading to Halotag-EGFP plasmid. The constructions coding for the two tandems between EGFP and tdimer2(12) or mRFP1 (2), named TdRed-EGFP or mRFP1-EGFP respectively, are generous gifts from Dr S. Ahmed (Center for Molecular Medicine, Biopolis Singapore). The cloning strategy enables us to maintain the same peptide linker (SGLRSRGDPPVAT) between the FRET partners for the mCherry-EGFP, EGFP-EGFP, mStrawberry-EGFP, mTFP1-EGFP, mTFP1-EYFP and Halotag-EGFP tandems.

The plasmids coding for EGFP-H4 and mTFP1-H4 were obtained by cloning histone H4 cDNA (IMAGE:2130477) in pEGFP-C1 (Clontech) and pmTFP1-C1 using EcoRI and KpnI sites of the MCS.

Lifetime data analysis

Using the time domain fluorescence lifetime microscope, the acquired fluorescence decays were deconvoluted with the instrument response function and fitted by a Marquardt nonlinear least-square algorithm using Globals Unlimited software (Laboratory for Fluorescence Dynamics; University of California, Irvine). Three different approaches were considered when performing the fits:

(i) A two species model in which two populations are taken into consideration (an interacting fraction corresponding to a population which relaxes through FRET (f_D) and a non-interacting fraction in which the donor lifetime remains undisturbed ($1-f_D$)). In this case the donor lifetime obtained out of the single exponential fit from cells expressing the donor alone was

assigned and fixed into the non-interacting fraction of the double exponential model for cells expressing the corresponding tandem (3,4).

$$i(t) = (1 - f_D)e^{-t/\tau_D} + f_D e^{-t/\tau_F} \qquad \text{(Eq.S1)}$$

In Eq.1 f_D stands for the fraction of interacting donor, τ_D is the fixed donor lifetime and τ_F is the discrete FRET lifetime.

(ii) Using the same formalism, it was also considered that instead of a discrete FRET lifetime, a distribution of lifetimes could arise from the different orientations between donor and acceptors. In this case we used a stretched exponential approach (5,6) for the FRET lifetime and discrete for the donor.

$$i(t) = (1 - f_D)e^{-t/\tau_D} + f_D e^{(-t/\tau_F)^{\beta}} \qquad \text{(Eq.S2)}$$

All parameters in Eq. 2 are already defined in Eq.1 but the coefficient β, which is related to the heterogeneity parameter of the sample.

(iii) The last considered approach was to leave both lifetimes free, still considering a discrete double exponential for the tandem. This system would account for the possibility of having two discrete FRET lifetimes out of the interaction between donor and acceptor (6).

$$i(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \qquad \text{(Eq.S3)}$$

In Eq.3 A_1 and A_2 are the pre-exponential factors (which in turn are related to the fraction of each FRET population) and τ_1 and τ_2 are the two FRET lifetimes.

For conventional bi-exponential analysis using fixed donor lifetime, the FRET efficiency, E, was calculated as follows:

$$E = 1 - \tau_F / \tau_D \qquad \text{(Eq.S4)}$$

where τ_D was the fixed fluorescence lifetime of the donor, and τ_F the shortened FRET lifetime.

The mathematical approach in order to recover the minimal fraction of interacting donor (mf_D) out of the diminution of donor mean lifetime alone and in the presence of acceptor was described elsewhere (4). Briefly, considering a two species system with single exponential

donor and taking into account the definition of mean lifetime, the fraction of donor in interaction (f_D) can be defined as

$$f_D = [1-(<\tau>/\tau_D)] / [1-(<\tau>/\tau_D)-(\tau_F/\tau_D)^2+(<\tau>/\tau_D)\times(\tau_F/\tau_D)] \qquad \text{(Eq.S5)}$$

In which $<\tau>$ represents the mean lifetime, τ_D stands for the donor lifetime and τ_F is the FRET lifetime. If we represent f_D as a function of τ_F/τ_D and $<\tau>/\tau_D$, a minimal value of f_D described for each value of τ_F/τ_D is revealed. The function which describes this minimum is the minimal fraction of donor undergoing FRET (mf_D). This means that f_D can be minimized following τ_F/τ_D. The partial derivative ($\partial f_D/\partial(\tau_F/\tau_D)$) is zero for $\tau_F = <\tau>/2$. Replacing τ_F by $<\tau>/2$ in Eq.5 gives

$$mf_D = [1-(<\tau>/\tau_D)] / [(<\tau>/2\cdot\tau_D)-1]^2 \qquad \text{(Eq.S6)}$$

Analysis of the data was done using imageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). Raw images were first smoothened by a 3x3 mask to decrease the noise. Calculation of the mean lifetime (4) was applied on background subtracted stacks of time-gated images. Eq.6 was then applied on mean lifetime images using fixed value of τ_D to recover mf_D.

RESULTS

f_D is influenced by the difference between donor and acceptor maturation rates

In order to find a possible explanation for the low f_D values recovered for most of the tandems analyzed we hypothesized that differences in donor and acceptor FP maturation rates might play a role. Different maturation rates for different FP's would lead to different situations, incomplete maturation of the donor, incomplete maturation of the acceptor or incomplete maturation of both, donor and acceptor. We are only concerned for the second situation since FRET-FLIM focuses on the variation of the donor fluorescence decay profile. Since tandems are constantly produced in the cell, we designed an experiment in which protein production is stopped, so that all tandems have the time to maturate.

We tested cells expressing mCherry-EGFP tandem treated with cycloheximide (100 μg/ml) for 1hr, 2hr and 4hr, respectively. Cycloheximide is known to block protein synthesis (7). We first controlled that cycloheximide did not affect EGFP lifetime by treating cells expressing EGFP alone for 1h, 2h and 4h. For all these conditions, the lifetime remained always at 2.60+/-0.01 ns (n = 30). Fig.S1 shows a representative experiment in which the fluorescence decay profile of EGFP alone is compared to the tandem mCherry-EGFP without drug and tandem mCherry-EGFP treated for 2 hours. It can be observed that FRET occurs for both conditions, as expected. But for the cell treated with cycloheximide the fluorescence decay is much faster than the cell without drug. This clearly shows the influence of cycloheximide upon f_D: 0.50+/-0.02 (n = 10) for 1hr treatment, 0.53+/-0.02 (n = 10) for 2hr treatment and 0.55+/-0.02 (n = 10) for 4hr treatment, to be compared to 0.45+/-0.02 (n = 10) without cycloheximide (Table 1). Slight increase in f_D is observed meaning that the difference in maturation rate plays a role in the fraction of unobserved acceptor. Note that E remained always constant for all experiments: E (mCherry-EGFP) = 0.58+/-0.02, E (mCherry-EGFP (1h treatment) = 0.56+/-0.08 (n = 10), E (mCherry-EGFP (2h treatment) = 0.58+/-0.05 (n = 10) and E (mCherry-EGFP (4h treatment) = 0.58+/-0.04 (n = 10).

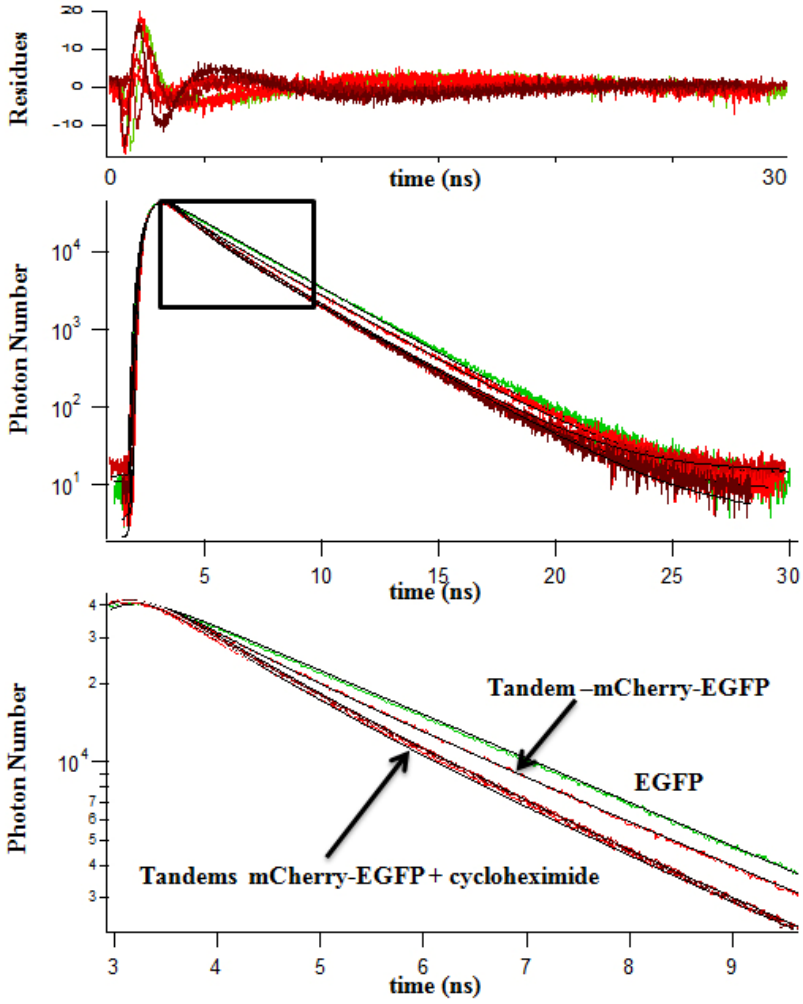

Figure S1. Effect of protein maturation on fD determination.
Middle panel: Fluorescence decay profiles for EGFP alone (green decays), mCherry-EGFP tandem with (dark red decays) or without (red decay) cycloheximide, in live He-La cells using a TSCSPC system, are fitted (black lines) with a single exponential (for EGFP alone) and a discrete double exponential (for tandem). The residues of these fits are presented in the upper panel. The lower panel focuses the beginning of the fluorescence decays (black square in the middle panel).

Confidence plot of the stretched exponential analysis

Two confidence plots using a double exponential stretched fit for mCherry-EGFP and mTFP1-EYFP are shown in Fig.S2 (discrete lifetime for the donor (τ_D) and stretched lifetime for FRET (β, τ_F)). For these two tandems only mTFP1-EYFP presents a minimal chi-square (χ^2) as a function of β, which is not the case for mCherry-EGFP. Interestingly, in the case of other tandems like mRFP1-EGFP, mStrawberry-EGFP, HaloTag(TMR)-EGFP and mTFP1-mOrange exhibiting also a low f_D values, it was not possible to find a minimum χ^2 as a function of β. The use of a stretched exponential that describes and quantifies FRET is here

unclear since χ^2 behaves linearly for values $0.7 < \beta < 1$. The confidence plot for mTFP1-EYFP, however, presented a minimum ($\beta = 0.7$ +/-0.01, n = 6) suggesting that, in this case, the stretched exponential analysis is an alternative to the discrete double exponential model (Table 2). Moreover, high f_D values (close to 1) are found using the stretched exponential approach opening the discussion of the occurrence and extent of a spectroscopic heterogeneity of the acceptor population and/or a certain distribution of the orientation between donors and acceptors.

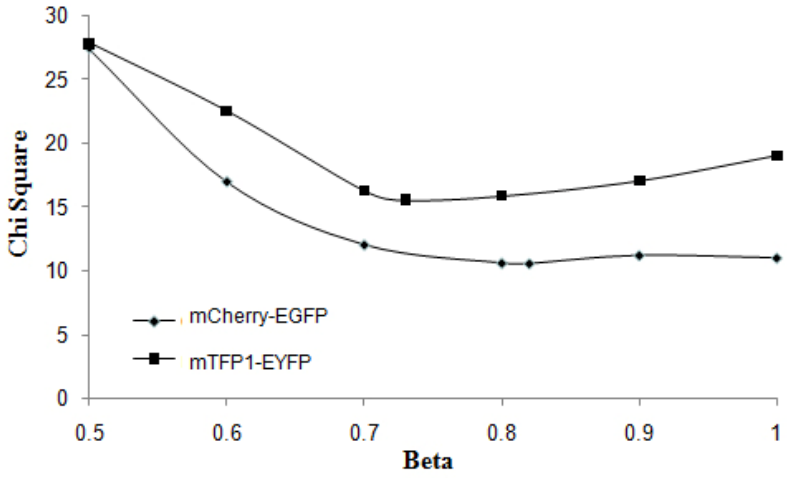

Figure S2. Confidence plot of mTFP1-YFP and mCherry-EGFP tandems using stretched exponential model.
Confidence plot for mCherry-EGFP (black circles) and mTFP1-EYFP (black squares) using a double exponential model, discrete for donor lifetime and stretched for the FRET lifetime. The confidence plot was obtained calculating the χ^2 values fixing the stretched parameter (β) to 0.5, 0.6, 0.7, 0.8, 0.9 and 1.

References

1. Tramier, M., M. Zahid, J.C. Mevel, M.J. Masse and M. Coppey-Moisan. 2006. Sensitivity of CFP/YFP and GFP/mCherry pairs to donor photobleaching on FRET determination by fluorescence lifetime imaging microscopy in living cells. Microsc. Res. Tech. 11:933-942
2. Campbell R.E, O. Tour, A.E Palmer, P.A Steinbach, G.S. Baird, D.A. Zacharias and R.Y. Tsien. 2002. A monomeric red fluorescent protein. Proc Natl Acad Sci U S A. 99:7877-7882.
3. Emiliani, V., D. Sanvitto, M. Tramier, T. Piolot, Z. Petrasek, K. Kemnitz, C. Durieux, and M. Coppey-Moisan. 2003. Low intensity two-dimensional imaging of fluorescence lifetimes in living cells. Appl. Phys. Lett. 83:2471-2473

4. Padilla-Parra, S., N. Audugé, M. Coppey-Moisan and M. Tramier. 2008. Quantitative FRET analysis by fast acquisition time domain FLIM at high spatial resolution in living cells. Biophys. J. 95:2976-2988
5. Lee, K.C., J. Siegel, S.E. Webb, S. Lévêque-Fort, M.J. Cole, R. Jones, K. Dowling, M.J. Lever and P.M. French. 2001. Application of the stretched exponential function to fluorescence lifetime imaging. Biophys J. 81:1265-1274
6. Wu, B., Y. Chen and J.D. Müller. 2009. Fluorescence fluctuation spectroscopy of mCherry in living cells. Biophys. J. 96:2391-404
7. Chu, C and A.J. Shatkin. 2008. Apoptosis and autophagy induction in mammalian cells by small interfering RNA knockdown of mRNA capping enzymes. Mol. Cell Biol. 28:5829-5836