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Practical Use of Corrected Fluorescence Excitation and Emission Spectra of Fluorescent Proteins in Förster Resonance Energy Transfer (FRET) Studies

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Corrected fluorescence excitation and emission spectra have been obtained from several enhanced variants of the green fluorescent protein (EGFP) isolated from the jellyfish *Aequorea victoria*, blue fluorescence protein (EBFP), cyan fluorescent protein (ECFP), EGFP and yellow fluorescent protein (EYFP–citrine) and from the red fluorescent protein (DsRed) isolated from the coral species *Discosoma*. The spectra are stored in a database. This report describes how the spectra can be used as templates to derive the critical transfer distance for any pair of fluorescent proteins.

KEY WORDS: Fluorescent proteins; FRET; GFP; DsRed; resonance energy transfer.

INTRODUCTION

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* together with its differently colored mutants has received widespread use as a natural, brightly fluorescent marker in cell biology [1–3]. Pairs of fluorescent proteins are widely used as donor–acceptor or Förster resonance energy transfer (FRET) pairs and are as such fused to the protein(s) of interest and genetically encoded in cells. FRET is a very powerful method for obtaining distance information on macromolecular complexes. It is based on the phenomenon that excited-state energy from a donor to an acceptor molecule is transferred nonradiatively through space [4]. Note that donor and acceptor molecular dipoles are very weakly interacting, which suggests that they can be neither nearest neighbours nor positioned at van der Waals distance from each other. FRET has been developed during the last decades as a spectroscopic ruler in the range of 2–7 nm to determine donor–acceptor distances in biological macromolecules (reviewed in, among others, references [5–9]). For a review of the principles of FRET applied to cell biology and microscopy, see references [10–14].

We collected corrected fluorescence excitation and emission spectra of several enhanced variants of green fluorescent protein (EGFP): blue fluorescence protein (EBFP), cyan fluorescent protein (ECFP), green fluorescent protein (EGFP), yellow fluorescent protein (EYFP–citrine), all from *Aequoria victoria*, and the red fluorescent protein from the coral species *Discosoma* (DsRed). From these spectra the parameters required for quantization of resonance energy transfer (overlap integral, critical transfer or Förster distance) can be easily recovered. We have made use of these spectra in various undergraduate and graduate courses to demonstrate and exercise the calculation of the spectral overlap integral and critical transfer distance. In this report, we give an account of this method.

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MATERIALS AND METHODS

The isolation of these proteins was performed as described in ref. [15]. The steady-state fluorescence spectra were obtained with a Spex-Fluorolog 3.2.2 (Horiba Jobin Yvon, Longjumeau, France) spectrofluorometer at room temperature (295 K). The concentration of all fluorescent proteins was in the range 50–100 nM and a quartz cuvette of 1.0 × 0.4 cm contained the protein solution. For all FPs except EBFP, the slits in the double-grating excitation and emission monochromators were 1 nm. For EBFP, these slits were 2 nm. Both excitation and emission spectra were corrected by a procedure supplied by the manufacturer and normalized to unity using the maximum values. The spectra were acquired at wavelength steps of 1 nm.

The rate of resonance energy transfer k_T is given by the Förster rate equation

$$k_T = (1/\tau_D^0)\,(R_0/R)^6 \quad (1)$$

where τ_D^0 is the donor fluorescence lifetime in the absence of acceptor, R is the actual donor-acceptor distance, and R_0 is the critical transfer distance (or Förster distance) at which the rate of transfer is equal to the fluorescence decay rate:

$$R_0^6 = 8.785 \times 10^{-5}\,\kappa^2 Q_D^0 J/n^4 \quad (2)$$

where κ^2 is the orientation factor between donor and acceptor molecules, Q_D^0 is the quantum yield of donor fluorescence in the absence of acceptor, and n is the index of refraction, usually taken as 1.4. J is the overlap integral between the fluorescence spectrum of the donor and the molar absorption spectrum of the acceptor:

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad (3)$$

where F_D is the peak-normalized fluorescence spectrum of the donor and ε_A is the absorption spectrum of the acceptor. The critical transfer distance R_0 is in units of angstroms, whereas λ is in nanometers and ε_A in $M^{-1}cm^{-1}$.

Equations (2) and (3) were programmed in Igor Pro (Wavemetrics Inc., Lake Oswego, OR).

RESULTS AND DISCUSSION

Corrected excitation and emission spectra of all FPs are shown in Fig. 1. From these spectra one can easily evaluate the critical transfer distance R_0 and the spectral overlap integral J provided that the quantum yield of the donor fluorescence Q_D^0 and the molar extinction coefficient of the acceptor at the wavelength of maximum light absorption $\varepsilon_A(\lambda_{max})$ are known. These data are known from the literature [1,16] and are summarized in Table I. A typical protocol for evaluation of transfer parameters in Igor Pro is given in the procedure section for the donor–acceptor pair ECFP–EYFP. After the data files are read in (emission spectrum of ECFP, excitation spectrum of EYFP, and the respective wavelengths), the emission spectrum of ECFP is divided by its integral, so that the integrated spectrum $[\int F_D(\lambda)d\lambda]$ is unity. Then, the excitation spectrum of EYFP is multiplied by its extinction coefficient (Table I) to yield the molar absorption spectrum of the acceptor. This is followed by the calculation of the spectral overlap integral J, in which integration is performed using a trapezoidal algorithm. Finally, the critical transfer distance R_0 is calculated according to Eq. (2) using the quantum yield $Q_D^0 = 0.39$ of CFP, the calculated J, the refractive $n = 1.4$, and the orientation factor $\kappa^2 = 1$ (antiparallel transition dipoles of donor and acceptor).

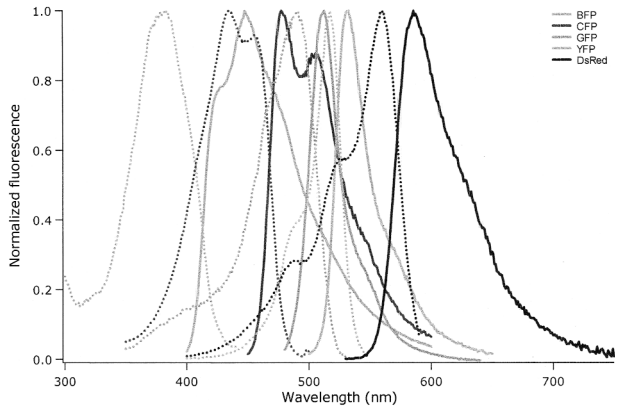

Fig. 1. Corrected excitation (dashed lines) and emission (solid lines) spectra of (from left to right) EBFP, ECFP, EGFP, EYFP, and DsRed.

Table I. Useful Numbers for Calculation of Spectral Overlap Integral and Critical Transfer Distance Between Fluorescent Proteins[a]

FP	Donor quantum yield (−)	Acceptor extinction coefficient (M^{-1} cm^{-1})
EBFP (F64L, Y66H, Y145F)	0.24	22,000
ECFP (F64L, S65T, Y66W, N146I, M153T, V163A)	0.39	32,500
EGFP (F64L, S65T)	0.64	56,000
EYFP–Citrine (S65G, V68, Q69K, S72A, T203Y)	0.61	62,000
DsRed	0.70	75,000

[a] The data for EBFP, ECFP, EGFP, and EYFP are from reference [1], the data for DsRed are from reference [16].

Table II. Numerical Values of Spectral Overlap Integral J and Critical Transfer Distance R_0 for Various Donor–Acceptor Pairs of Fluorescent Proteins

Donor–acceptor pair	J (M^{-1} cm^3)	R_0 ($\kappa^2 = 1$) (Å)	R_0 ($\kappa^2 = 4$) (Å)
EBFP → EGFP	1.24614×10^{15}	44	55
ECFP → EGFP	1.60525×10^{15}	49	62
ECFP → EYFP	1.55479×10^{15}	49	62
EGFP → EGFP	1.02816×10^{15}	49	62
EGFP → EYFP	2.30938×10^{15}	57	71
EGFP → DsRed	2.88576×10^{15}	59	74
EYFP → EYFP	9.76207×10^{14}	49	62
EYFP → DsRed	3.92001×10^{15}	62	78

In this way, all parameters for various donor–acceptor pairs have been evaluated from the available spectra and data; these values have been collected in Table II. We have also listed in Table II the R_0 values when the maximal value of the orientation factor is reached, namely for parallel transition dipoles ($\kappa^2 = 4$). Some interesting conclusions can be drawn from the data in Table II. The more red-shifted the absorption spectra, the higher are the molar extinction coefficients (Table I). In addition, the more red-shifted the fluorescence spectrum, the higher is the quantum yield (Table I). Therefore, the overlap integrals exhibit a steady increase when the red-shifted fluorescent proteins act as acceptor. This increase is reflected as well in a larger critical distance R_0. Homo-transfer between different EGFPs (or between different EYFPs) can occur quite readily, as judged from the appreciable J and R_0 values. Homo-transfer of GFP fusion proteins in cells has been described recently via fluorescence anisotropy decay measurements [17].

PROCEDURE

The annotated procedure for Igor Pro was used to calculate the overlap integral and critical transfer distance from experimental fluorescence data.

```
General text load from
   "cfpem.dat"                  loading in experimental data
Data length: 151,
   waves: cfpem                 (151 data points per file)
General text load from
   "wlcfp.dat"
Data length: 151,
   waves: wlcfp
General text load from
   "yfpex.dat"
Data length: 151, waves:
   yfpex
General text load from
   "wlyfpex.dat"
Data length: 151, waves:
   wlyfpex
•display cfpem vs
   wlcfp                        make graph of 2 spectra
•append yfpex vs
   wlyfpex
•duplicate cfpem
   cfpintegr                    duplicate in order not to
                                   overwrite original
                                   data
•duplicate wlcfp
   wlcfpmod
•duplicate yfpex
   yfpexnorm
•duplicate wlyfpex
   wlyfpexmod
•duplicate cfpem
   cfpemnorm
•integrate/T cfpintegr          integration of emission
                                   spectrum
•edit cfpintegr                 get value of integral
•cfpemnorm=cfpemnorm/
   68.1649                      division by value of
                                   integral
•yfpexnorm=yfpexnorm*
   62000                        multiplication with
                                   ε_max
•edit wlcfpmod,cfpemnorm,
   wlyfpexmod,yfpexnorm
•DeletePoints 0,50,
   yfpexnorm, wlyfpexmod        shift of absorption
                                   spectrum such
•duplicate wlyfpexmod              that spectra start at
   overlap                         same λ
•overlap=cfpemnorm*
   yfpexnorm*
   wlyfpexmod^4                 overlap integral J
                                   (eq. 3)
•duplicate overlap overla-
   pint
•integrate/T overlapint         integration of J
•edit overlapint                get value of J
•make/d rzero
•rzero=8.785e-5*1*0.39*
   1.55479e15/(1.4^4)           calculation of R0
                                   [Eq. (2)]
•rzero=rzero^0.1666666
•display rzero                  get value of R0
```

CONCLUSION

When the corrected excitation and emission spectra of different fluorescent proteins are used, it is rather straightforward to recover the correct energy transfer parameters. All spectra in digital form are available upon request.

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