Journal of Microscopy, Vol. 217, Pt 3 March 2005, pp. 200–204
Received 19 November 2003; accepted 5 October 2004

Two-photon excitation and emission spectra of the green fluorescent protein variants ECFP, EGFP and EYFP

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Key words. GFP, pH, spectra, two-photon excitation.

Summary

Two-photon (TP) excitation (820–1150 nm) and emission (280–700 nm) spectra for the fluorescent proteins (FPs) ECFP[3], EGFP[3] and EYFP[3] produced in human tumour cells were recorded. TP excitation spectra of pure and highly enriched samples were found to be more differentiated in comparison with their one-photon (OP) spectra. They exhibited more pronounced main and local maxima, which coincided among different purity grades within small limits. TP and OP emission spectra of pure and enriched samples were identical. However, in crude samples, excitation was slightly blue-shifted and emission red-shifted. The data indicate that both OP and TP excitation routes led to the same excited states of these molecules. The emission intensity is dependent on the pH of the environment for both types of excitation; the emission intensity maximum can be recorded in the alkaline range. Reconstitution of emission intensity after pH quenching was incomplete, albeit that the respective spectral profiles were identical to those prequenching. When emission data were averaged over the whole range of excitation, the resulting emission profile and maximum coincided with the data generated by optimal excitation. Therefore, out-of-maximum excitation, common practice in TP excitation microscopy, can be used for routine application.

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[3]The discovery of numerous fluorescent proteins led to the introduction of a specific nomenclature (Labas *et al.*, 2002) according to which the *Aequorea victoria* fluorescent protein derivatives are named GFP. EGFP and others are commercial product names (BD Biosciences Clontech, Palo Alto, CA, U.S.A.).

Introduction

Multiphoton microscopy has evolved in the past decade (Denk *et al.*, 1990) and is becoming a powerful tool in biomedical research (Piston, 1999; König, 2000; So *et al.*, 2000; Brown *et al.*, 2001; Williams *et al.*, 2001; van Roessel & Brand, 2002). Although the method is restricted to fluorescence microscopy, its features make it attractive to the study of living cells, tissue sections or even tissues *in situ* particularly in combination with various fluorescent proteins (FPs). These proteins were first discovered in *Aequorea victoria* (green fluorescent protein, GFP) and later in other species (Labas *et al.*, 2002). Variants thereof were constructed (Heim *et al.*, 1995; Patterson *et al.*, 1997; Yang *et al.*, 1998; Matz *et al.*, 1999; Gurskaya *et al.*, 2001, 2003; Fradkov *et al.*, 2002; Chudakov *et al.*, 2003). Immediately after discovery, these proteins became exciting tools for many approaches to the study of basic biological problems. Owing to the molecular structure of the fluorochromes, the photophysics of these proteins is extremely complex and leads to broad one-photon (OP) excitation spectra (Chattoraj *et al.*, 1996; Weber *et al.*, 1999). For two-photon (TP) excitation, or multiphoton excitation in general, further complexity may arise from physical peculiarities such as different selection rules and the effects of vibronic coupling (Xu *et al.*, 1996). In theory, similar emission characteristics of fluorochromes for both OP and TP excitation are predicted, but in practice they may differ significantly (Albota *et al.*, 1998). Detailed knowledge of the spectra is therefore important, especially in multicolour labelling of biological specimens. TP excitation to states that correspond to local maxima of a spectrum might be efficiently used by tuning the laser wavelength appropriately. For certain FPs produced in *Escherichia coli*, TP spectra or spectral properties have already been investigated (Xia *et al.*, 1999; Heikal *et al.*, 2000). Here

we present TP excitation and emission data in direct comparison with their OP equivalents for the frequently used FP variants, enhanced cyan/green/yellow fluorescent protein (ECFP, EGFP and EYFP), produced in a human tumour cell line.

Materials and methods

Preparation of protein samples for spectroscopic measurements

FPs (ECFP, EGFP and EYFP) were produced in our laboratory in cell clones of a human tumour cell line (LCLC-103H; DSMZ #ACC384, Braunschweig, Germany) overexpressing the respective FP using methods described by Bestvater *et al.* (2002a). Measurements were carried out on samples of three different degrees of purity: (i) crude cell extracts (CS) were prepared by sonication of cell suspensions in phosphate-buffered saline (pH 7.2) or – in the case of pH variation experiments – in McIlvaine buffer (McIlvaine, 1921) of the respective pH. Coarse material was removed by centrifugation at 15 000 ***g***. The suspensions obtained were stored until use at −80 °C. In the pH recovery experiments, the pH was titrated to that required by appropriate volumes of either 1 N NaOH or 1 N HCl. The respective FPs were purified close to homogeneity by exclusion centrifugation with Microcon 10 and 100 filters (Millipore, Eschborn, Germany) and by ion exchange chromatography with HighQ Resin (Bio-Rad, München, Germany). A 50 mM Tris, pH 8.5, buffer system and a discontinuous 50–130–300 mM NaCl gradient were used for elution. These samples were (ii) enriched (ES) to either 50–70% or (iii) pure FPs (PS) as assumed from Coomassie-stained polyacrylamide gels or OP absorption data.

OP spectra

Absorption spectra were measured with a CaryE 4 spectrophotometer (Varian, Mulgrave, Australia). The OP fluorescence spectra were recorded on a SLM-Aminco 8100 fluorimeter (SLM, Urbana, IL, U.S.A.) equipped with a 150-W xenon lamp and double grating monochromators (4 nm slit width). The spectral data were corrected with respect to the power distribution of the illumination source, the detector response and the buffer signal.

TP spectra

A Ti: sapphire laser with 45-fs pulses at 804 nm and a pulse energy of 1 mJ at a repetition rate of 1 kHz and an optical parametric amplifier (TOPAS) were used to tune the wavelength continuously between 820 and 1150 nm as described in detail by Bestvater *et al.* (2002b). Briefly, fluorescence was collected by a multimode fibre that was coupled to a grating spectrometer. Excitation spectra were compiled by signal integration within the limits of the recorded fluorescence spectrum. They were subsequently corrected for laser power, which was either measured before and after recording a set of spectra or measured simultaneously with each individual spectrum, respectively. The curves were smoothed by a floating mean function (n = 3 nm). Different samples of the individual FPs were measured in several independent sessions. The reliability of the measurements was ensured by careful calibration of the instrumental parameters.

Results and discussion

Excitation and emission spectra of EGFP

The FP was extracted from a clone of human tumour cells that expressed it in abundance. FPs are difficult to purify because of their tendency to gelatinize upon an increase in concentration. Gelatinization enhances the light scattering of the samples considerably and interferes with measurements. Crude cell extracts were used as well as pure and enriched specimens, because they represent the cytoplasm within cells much better than pure samples. Excitation and emission spectra were recorded over a range of 820–1150 nm and 280–700 nm, respectively; in the figures presented here, only the relevant information is shown. Pure and enriched samples revealed TP excitation spectra with almost overlapping profiles irrespective of the degree of purity (Fig. 1a). A characteristic profile with a bandwidth of about 200 nm, local maxima at 904 and 934 nm and a main peak at 970 nm appeared. The TP profile is similar to the OP spectrum but the main peak is slightly blue-shifted and the local maxima are more emphasized. The spectrum of the crude extract is even more blue-shifted. The TP emission spectra of the pure and enriched samples match perfectly (Fig. 1b); the maximum appears at 511 nm irrespective of whether an averaged profile or a single spectrum at maximal excitation was plotted. The OP emission spectrum is also identical to the TP spectrum of the pure preparation. The TP spectrum of the crude extract is clearly red-shifted. Local and main maxima as well as OP and TP data from the literature (Blab *et al.*, 2001; Patterson *et al.*, 2001; Labas *et al.*, 2002) are compiled in Table 1. Excitation data differ within reasonable limits, but emission data are always equal. Differences may arise from technical conditions (instrumentation, data normalization) and from the sample itself (e.g. expressing cell type and purification procedure).

The excitability and the fluorescence yield of GFP and the enhanced variants thereof strongly depend on the pH of the environment (Patterson *et al.*, 1997; Haupts *et al.*, 1998; Wachter *et al.*, 1998; Elsliger *et al.*, 1999; Weber *et al.*, 1999; Baird *et al.*, 2000; Heikal *et al.*, 2000). Maximum emission is obtained at non-physiological, high pH. The sensitivity to pH is attributed to possible charge states of the chromophore, among which the neutral and anionic states are relevant for fluorescence (Elsliger *et al.*, 1999; Weber *et al.*, 1999). However, we cannot rule out that, in addition, extremely low pH or extended exposure to low pH may also influence the folding of

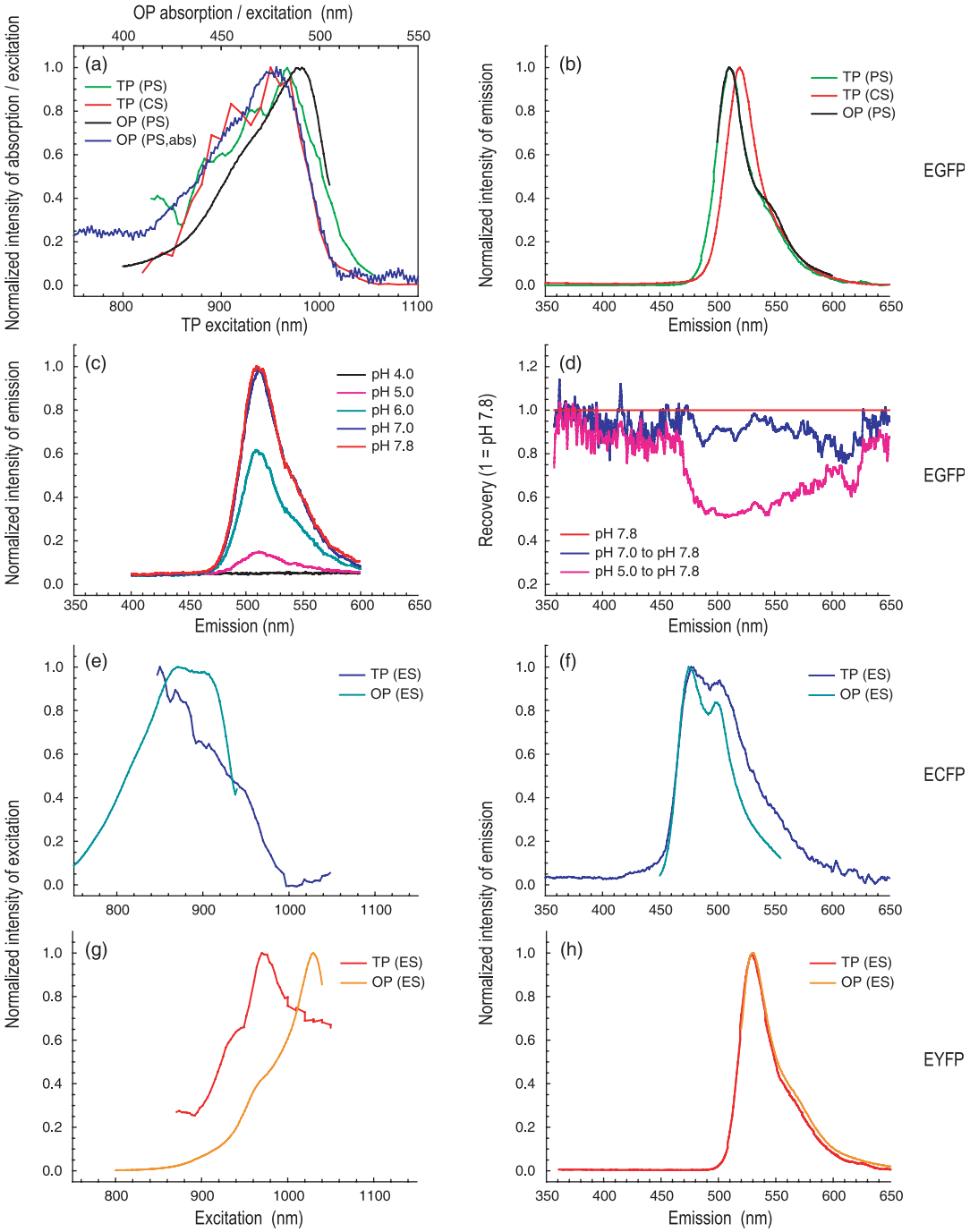

Fig. 1. TP and OP spectra of EGFP (a–d), ECFP (e,f) and EYFP (g,h). (a) TP excitation and OP absorption profiles of EGFP: pure sample (PS) TP; crude sample (CS) TP; pure sample (PS) OP; and pure sample (PS) OP absorption (abs). The profiles correspond to each other; however, in comparison with OP excitation they are blue-shifted. (b) The normalized emission data generated by TP and OP excitation fit together for the purified sample; only the profile generated by TP excitation of the crude sample is red-shifted. (c) Quenching of EGFP emission by pH variation. The reference sample is pH 7.8 (= 1). The profile was generated by excitation at 895 nm. At pH 4.0 only a residual intensity remained. (d) Recovery of emission after pH quenching was complete for the pH 7 to pH 7.8 transitions but reached only about 50% for the pH 5.0 to pH 7.8 transitions. ECFP profiles (e,f): excitation profiles for an enriched sample (ES). The TP-generated emission profile is a mean calculation over the total excitation range, and correlates well with the OP-generated spectrum. EYFP spectra (g,h): TP and OP excitation spectra of a sample enriched (ES) for EYFP. The TP-generated emission profile of the enriched sample is the mean calculated over the total excitation range, and coincides with the OP-generated profile.

Table 1. Comparison of OP/TP excitation and emission data.

	OP maxima			TP maxima		Excitation max. shift	Emission max. shift	TP 850 nm excitation
	Abs. (nm)	Ex. (nm)	Em. (nm)	Ex. (nm)	Em. (nm)	OPE→TPE (nm)	OPE→TPE (nm)	Yield (Em_{850}/Em_{max})
ECFP$^{(ES)}$	444	436	475	850	477	−11	+2	
ECFP$^{(ref.)}$		434*‡	472*	860‡				
EGFP$^{(PS)}$	488	491	510	970	511	−4	±0	
EGFP$^{(ES)}$	489	492	510		511		±0	0.32
EGFP$^{(CS)}$		488	510	950	521	−14	+11	
EGFP$^{(ref.)}$		489*‡ 471†	508*†	920‡				
EYFP$^{(ES)}$	514	515	530	970	530	−29	±0	
EYFP$^{(ref.)}$		514*‡	527*	960‡				0.33

CS, crude sample; ES, enriched sample; PS, pure sample; ref., reference.
*Patterson *et al.* (2001); †Labas *et al.* (2002); ‡Blab *et al.* (2001).

the protein and that this might result in an incomplete reconstitution and quenching of fluorescence. We investigated the pH dependence of EGFP emission after TP excitation in cell extracts at 895 nm excitation (Fig. 1c). Considerable quenching of signal intensity took place at acidic pH; at pH 4.0 less than 5% of the fluorescence at pH 7.8 remained. The flat profile of the pH 4 sample exhibited a maximum at 528 nm; this shift is probably a consequence of protonation. The recovery of emission from pH 7.0 and 5.0, respectively, to pH 7.8 was also investigated (Fig. 1d). Cell extracts were prepared within the respective pH conditions and the samples were titrated to pH 7.8 immediately before recording the spectra. The recovery from pH 7.0 to 7.8 was complete within the limits of experimental conditions. The recovery after transition from pH 5.0 to 7.8 was incomplete, only to about 50% of the prequenching level. We did not observe any emission shift for the recovered maxima after pH quenching. Comparable results were obtained in OP control measurements.

Excitation and emission spectra of ECFP and EYFP

Spectra were taken from enriched or crude samples of ECFP (Fig. 1e,f) and EYFP (Fig. 1g,h). The TP excitation spectrum of the sample enriched for ECFP must extend further in the region below 800 nm that was not measured. The excitation spectrum of ECFP- and of EYFP-enriched samples shows a blue shift relative to the OP spectrum of each. OP and TP emission spectra match for each protein. Emission spectra of ECFP overlap in profile and exhibit a main and a local maximum. A red shift in TP excitation was observed in comparison with the OP excitation spectrum. Samples of EYFP show identical mono-phasic TP and OP emission spectra. All peak data are compiled in Table 1.

In general, our TP measurements show a variation in the different excitation spectra; peaks and local maxima are shifted to some extent and intensities vary. In comparison with the OP spectra, TP spectra show a tendency to blue-shift. Emission spectra of purified FPs overlap clearly. Red shifts were observed in OP excitation of crude samples. In OP spectroscopy a red shift in emission spectra is known to depend on sample purity (Lakowicz, 1999). It appears that TP excitation/emission is also sensitive to this phenomenon. Calculation of the mean spectra for EGFP emission and their comparison with the respective spectrum taken at the maximum of excitation revealed coincidence, yet there were differences in fluorescence yield. It is generally impractical and time consuming to flush the TP laser cavity with dry nitrogen to achieve stable longer wavelength output (> 900 nm). Our findings imply that excitation with wavelengths that are even far below the excitation peak still generate reliable emission data.

Acknowledgements

We wish to thank Christina Barther for critical reading of the manuscript. The project was supported by the BMBF grants 13N744/5 to H.A. and 13N7308/6 to T.F. and the EU Biomed 2 Program grant PL963469 to H.A.

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