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Two-photon excitation action cross-sections of the autofluorescent proteins

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Abstract

We report on the values of the two-photon excitation action cross-sections of commercially available enhanced cyan, green, yellow, and red fluorescent proteins. The two-photon absorption spectra are very similar in shape to those measured for one-photon absorption. However, they exhibit a significant blueshift, which is attributed to the participation of a vibrational mode in the two-photon absorption process. The two-photon spectra are compared to that of flavine mononucleotide, which constitutes the main source of autofluorescence in mammalian cells. The definition of a relative detection yield between the autofluorescent proteins and flavine allows us to quantify the applicability of autofluorescent proteins in two-photon single-molecule studies in living cells. © 2001 Published by Elsevier Science B.V.

1. Introduction

Major innovations in recent years have largely revolutionized the fluorescence imaging of biological samples. Two-photon excitation using near-infrared wavelength short laser pulses increased the achievable penetration depth in thick samples, which for the first time renders imaging of thick tissues possible [1,2]. In addition, the out-of-focus photodamage of the samples is essentially avoided which further facilitates reliable volume imaging of fluorescent samples. Another advancement came from the discovery of the autofluorescent proteins originating from the jellyfish *Aequorea victoria* [3] ([4] and references therein) and the coral *Discosoma* sp. [5]. By fusing a specific protein with an autofluorescent protein, the functional imaging of complex processes in cells became possible for the first time: the role of microtubules in mitosis [6], functional oligomerization of cellular proteins [7], or changes in the intracellular Ca^{2+} level [8,9]. One advantage of the

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fluorescence labelling technique in comparison to bioconjugation is its extremely high sensitivity which makes experiments feasible down to the level of a single molecule, opening a new avenue for cell biological and biophysical research.

While the applicability of the autofluorescent proteins for single-molecule research in vivo has recently been demonstrated [7,10,11], its general application is still in early development. One major issue is the detectability of a single fluorophore within the autofluorescence background inside a living cell. The main component of this autofluorescent background is flavine molecules which are present at particularly high abundance of 100–1000 molecules per focal volume element [12]. Due to the broad absorption of flavine in the blue/green part of the visible spectrum the excitation spectra of the cellular autofluorescence strongly overlaps with the excitation spectra of the fluorescent proteins. For this reason, only the two most redshifted varieties, the enhanced yellow fluorescent protein (eYFP) and the red fluorescent protein (DsRed), provide a significantly stronger wavelength discrimination compared to the flavines in wide-field single-molecule applications [10].

Here we report on the prospects of using two-photon excitation (TPE) in order to largely increase the signal-to-background ratio in our quest for general single-molecule detection of the autofluorescent proteins in live cells. The basic idea for two-photon excitation rests on the earlier observation that the two-photon absorption cross-section of a variety of fluorescent molecules scales super-linearly with the one-photon absorption cross-section [13]. Hence, the ratio of the effective excitation rate of a fluorophore with high one-photon absorption cross-section, like the fluorescent proteins, and a fluorophore with a low one-photon absorption cross-section, like flavines, will be largely increased for two-photon excitation. Indeed our data show a super-linear scaling behaviour which has been previously described only for small organic molecules. For exploitation and optimization of the two-photon excitation scheme we have fully characterized and compared the two-photon spectroscopic properties of commercially available autofluorescent proteins and that of flavine mononucleotide. Our findings might lead to a novel strategy of two-photon imaging of single molecules using the autofluorescent proteins as markers.

2. Experimental

2.1. Sample preparation

The autofluorescent proteins were purified as described previously [10]. In brief, plasmids containing the coding sequences of the autofluorescent proteins with a C-terminal his_6 tag (peXFP, Clontech) were transformed into *E. coli* and cultured at 37 °C. The cells were harvested, lyzed and the fluorescent protein was extracted using a column of chelating sepharose (Pharmacia Biotech). Concentrations of the fluorescent proteins were determined by measuring their absorption spectra. SDS-PAGE analysis revealed a correct molecular weight and an estimated purity of at least 95%. 25 μl of stock solution of the fluorescent proteins and a stock solution of flavine mononucleotide were diluted in phosphate-buffered saline (PBS: 150 mM NaCl, 15 mM Na_2PO_4, pH 7.4) to obtain a final concentration in the low μM range. Fluoresceine was diluted from stock into distilled water (NaOH to adjust for $pH > 11$). All fluorophore concentrations were controlled spectroscopically.

2.2. Two-photon excitation

Femtosecond pulses from a Ti:sapphire laser (Tsunami, Spectra Physics) with a mean power of 1–200 mW (150 fs pulsewidth, 80 MHz repetition rate) were focussed by a 10× objective (Zeiss) into a quartz cuvette containing the solution. The focal spot had a diameter of approximately 100 μm. The fluorescence signal was discriminated from the excitation light by a 6 mm thick blueglass filter (BG, Schott) and detected on an avalanche photodiode (SPCM-141, EG&G) under an angle of 90°. The overall detection efficiency of the entire setup was about 1%. Fluorescence signals were integrated over periods of 50 ms.

2.3. Measurement of two-photon action cross-sections

In this study we report on the TPE action cross-section σ_{TPE}, defined as the product of the two-photon absorption cross-section, $\sigma^{(2)}$, and the fluorescence quantum yield, Φ. All samples were measured at mean laser powers ranging from 1–200 mW (1–212 MW/cm^2 peak intensity), and up to 400 mW. The data were corrected for the background signal, $F_0 < 10$ kcps, which was obtained from the pure buffer solutions on excitation. Since the measurement of absorption cross-sections in the two-photon case heavily depends on the spatial and temporal coherence of the excitation beam [14] a calibration against a reference spectrum was employed using the routine described in detail in [15]. In short, the fluorescence signal, F, was measured for the particular autofluorescent protein under study and compared to the fluorescence signal, F^{cal}, obtained from fluoresceine for which the two-photon action cross-section, σ^{cal}_{TPE}, has been reported [15]:

$$\sigma_{TPE} = \frac{\eta^{cal}}{\eta}\frac{c^{cal}}{c}\frac{F - F_0}{F^{cal} - F_0}\sigma^{cal}_{TPE}.$$

The collection efficiencies, η ($\eta = 0.5\%$ for DsRed, $\eta = \eta^{cal} = 1.1\%$ for all other fluorophores), and the respective sample concentrations, c, as obtained by one-photon absorption measurements on each sample were taken into consideration. For wavelengths > 960 nm, where values for fluoresceine were lacking, the corrections were extrapolated from the 960 nm calibration value. The calibration procedure ensures that any effects such as the strong influence of spatial and temporal coherence of the excitation beam on the obtained results can be neglected.

3. Results and discussion

The foremost characteristic of two-photon induced fluorescence is the dependence of the signal on the square of the excitation intensity [16]. For each wavelength measured we have recorded a power series of the detected fluorescence signal for peak intensities between 1 and 424 MW/cm^2 (Fig. 1). For all 84 experiments we found that the fluorescence signal, F, followed a power-law dependence on the excitation power, $F \propto I^{\alpha}$ with an average exponent of $\alpha = 1.92 \pm 0.17$ (mean $\pm$ SD). At the respective two-photon absorption maxima of the autofluorescent proteins, and thus at the highest signal-to-noise ratios, the average exponent was $\alpha = 2.00 \pm 0.03$ (Fig. 1). Hence, it can be safely concluded that the detected signals originated from a two-photon excitation process.

The TPE action cross-section spectra of the various autofluorescent proteins are given in Fig. 2 (data points) and are summarized in Table 1. The maxima of the two-photon absorption spectra of the cyan (eCFP), green (eGFP), and yellow (eYFP) fluorescent proteins at $\lambda_{max} = 860$, 920 and 960 nm, respectively, all fall in the tuning range of a standard Ti:sapphire laser equipped with broadband optics. The absolute values of the two-photon action cross-section, σ_{TPE}, at the maxima vary between 8 and 40 GM (1 GM =

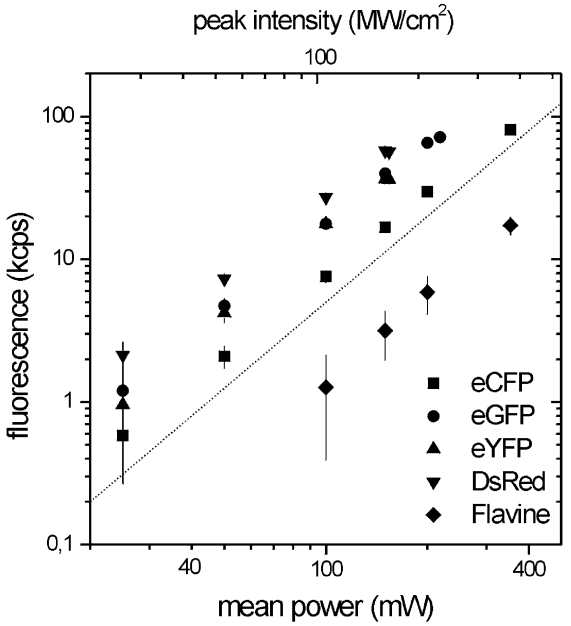

Fig. 1. Dependence of the fluorescence signal on the excitation intensity for the autofluorescent proteins at their peak two-photon absorption wavelength; eCFP: 860 nm, eGFP: 920 nm, eYFP: 960 nm, DsRed: 980 nm, and flavine mononucleotide: 760 nm. The fluorescence follows a power-law dependence on the intensity with a mean exponent of 2.00 ± 0.03 (dotted line).

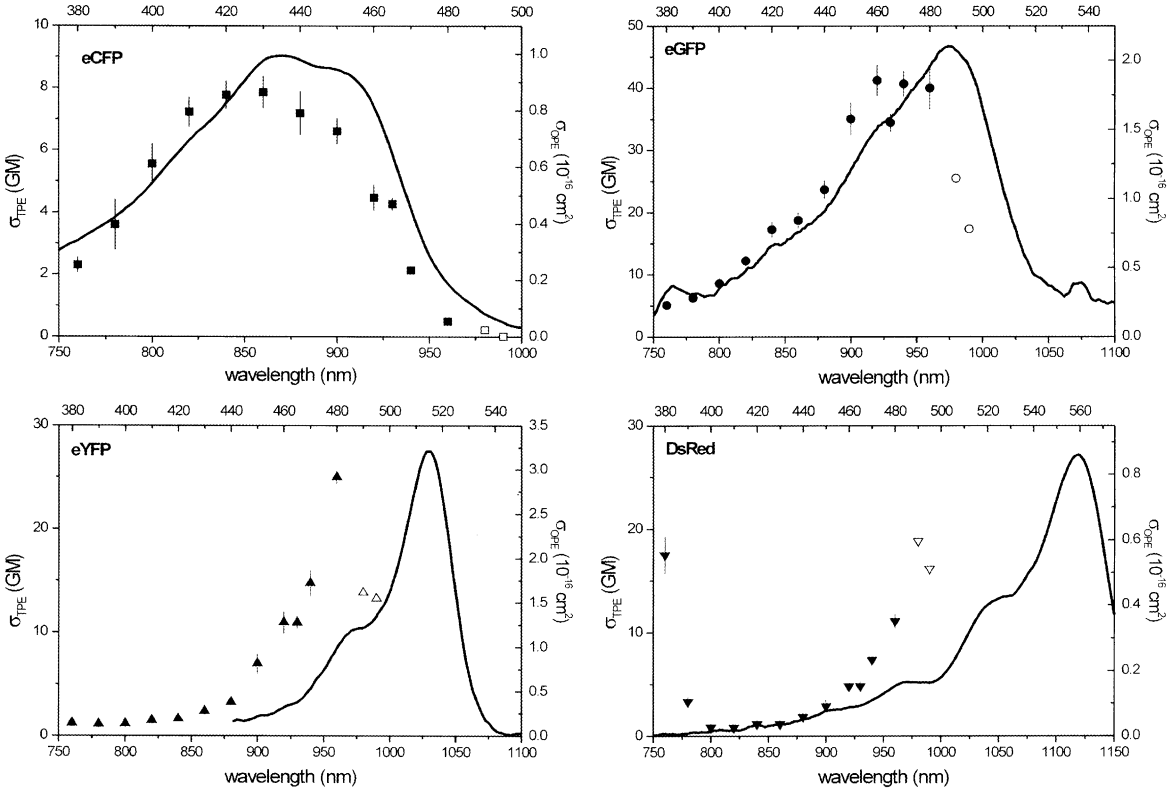

Fig. 2. Absolute TPE action cross-sections (σ_{TPE}, left axes) of eCFP, eGFP, eYFP, and DsRed in phosphate buffer (wavelength scale bottom). The values for $\lambda \leqslant 960$ nm were calibrated against the values for fluoresceine (pH > 11) [15] (filled symbols). For $\lambda > 960$ nm (open symbols) the calibration for 960 nm was extrapolated. The one-photon absorption cross-sections (σ_{OPE}, right axes) are shown for comparison (solid lines; wavelength scale top).

Table 1
Maximal values of the one-photon absorption (σ_{OPE}), and the TPE action cross-sections (σ_{TPE}; 1 GM = 10^{-50} cm^4 s/photon) of the commercial autofluorescent proteins and of flavine mononucleotide (FMN)

Fluorophore	One-photon excitation		Two-photon excitation		
	λ (nm)	σ_{OPE} (10^{-16} cm^2)	λ (nm)	σ_{TPE} (GM)	Shift (cm^{-1})
eCFP	434	0.99	860	7.87	255
eGFP	489	2.10	920	41.21	773
eYFP	514	3.21	960	24.98	1290
DsRed	558	0.86	960	11.01	n.d.
FMN	266	1.22 [27]	–	–	n.d.
	373	0.40	760	0.39	
	445	0.48	–	–	

Blueshift of the two-photon spectra with the respect to the one-photon spectra.

10^{-50} cm^4 s/photon). The limited tuning range of our standard Ti:sapphire system did not allow for the identification of the maximum of the two-photon spectrum for DsRed, predicted to be around 1100 nm. The highest value obtained for DsRed was 20 GM at the wavelength of 980 nm. It should be noted that the spectra and absolute values of the TPE action cross-sections reported

here on eGFP are in good agreement to the values previously reported [2], and those of eYFP closely resemble the results obtained for a non-commercial, improved YFP mutant Citrine [17].

For the most redshifted autofluorescent protein, DsRed, current data suggest that it forms tetrameric complexes even at low concentrations [18] which, in turn, renders the definition of a molar absorption ε and thus the actual concentration in our experiment difficult [17,19]. For the data presented the values of ε given by the manufacturer were used (Table 1) [5,20]. The increase of the TPE action cross-section at wavelengths below 760 nm, for which a sub-quadratic power dependence ($\alpha = 1.68 \pm 0.12$) was found, does not correspond to any increase in the one-photon spectrum below 380 nm. This new component has recently been described by Marchant et al. [21] as an irreversible 'greening' of the protein by a three-photon transformation process, which might also explain the deviation from the expected quadric power dependence. Such higher-order photobleaching processes have been established before for a variety of other fluorophores [22].

Fig. 2 is subsequently used for a quantitative comparison of the one-photon (solid lines, top wavelength axis) with the two-photon absorption cross-section spectra (points, bottom wavelength axis). The two-photon spectra roughly follow the shape of the one-photon spectra (when dividing the wavelength scale by two). However a significant blueshift in excitation is recurrently observed for the two-photon excitation spectra in comparison to the one-photon spectra [1,15]. Whereas the absorption spectra are shifted the fluorescence emission spectra stay unchanged for one- and two-photon excitation [23,24], as predicted from Kasha's rule. The blueshift in the absorption spectrum is probably due to an additional, low-symmetry vibrational mode which couples to the electronic transition in order to get sufficient oscillator strength for the two-photon absorption process. For the pure electronic (0–0) transition the two-photon absorption process is parity-forbidden. We quantified the blueshift for the autofluorescent proteins by a least-square fit which overlays the one- and the two-photon spectra. The values for the blueshift found in this way are 255, 773 and 1290 cm^{-1} for eCFP, eGFP and eYFP, respectively. It is interesting to note that vibrational modes of 222, 775 and 1507 cm^{-1} have been identified for wild-type GFP by low-temperature line-narrowing experiments [25]. The different shifts found for the various autofluorescent proteins point to the appealing conclusion that depending on the fluorophore a different vibrational mode couples to the two-photon excitation process. However, it can not be excluded likewise that the different shifts are mediated by a change in the protein environment.

The absolute TPE action cross-sections as determined for the autofluorescent proteins (Fig. 2) and the flavine mononucleotide (Fig. 3) determined here are used to quantify the relative detection yield of the autofluorescent proteins in comparison to flavines necessary for single-molecule studies in living mammalian cells. Following a strategy developed by Harms et al. [10] we define the excitation-wavelength-dependent relative detection yield for the autofluorescent proteins as

$$R_{\mathrm{OPE/TPE}}(\lambda) = \frac{\eta\sigma(\lambda)}{\eta_{\mathrm{flavine}}\sigma_{\mathrm{flavine}}(\lambda)},$$

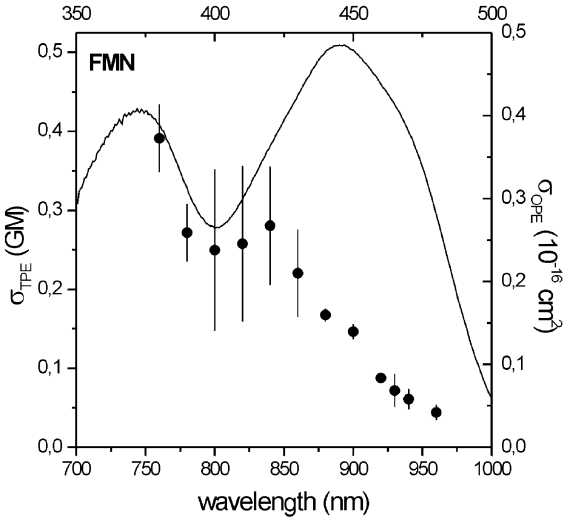

Fig. 3. Absolute TPE action cross-sections (σ_{TPE}, left axis) of flavine mononucleotides in phosphate buffer (symbols). The one-photon absorption cross-section (σ_{OPE}, right axis) is shown for comparison (solid line; wavelength scale top).

Table 2
Relative detection ratio of the autofluorescent proteins versus flavine mononucleotide for one- (R_{OPE}) and two-photon excitation (R_{TPE})

Fluorophore	One photon		Two photon	
	λ (nm)	R_{OPE}	λ (nm)	R_{TPE}
eCFP	435[a]	3.4	860	36
	457[b]	1.8		
eGFP	488[a,b]	8.7	920	467
eYFP	514[a,b]	405	960	566
DsRed	532[b]	$>10^4$	960	251

Values of R_{OPE} were taken from [10]. The value of R_{TPE} for DsRed will be considerably higher for its maximum absorption at wavelengths above 1000 nm.
[a] Value for excitation at the peak absorption.
[b] Value for wavelengths associated with the commonly used laser lines.

where the experimental detection efficiencies for the various fluorophores, η, and the absorption cross-sections, σ_{OPE} and σ_{TPE}, for the one- or two-photon case are considered. In the case of two-photon excitation $\eta/\eta_{flavine}$ was close to unity. The values for one- and two-photon excitation are summarized in Table 2. In the one-photon case the low values of relative detection yield for eCFP and eGFP ($R_{OPE} < 10$) render their use for wide-field single-molecule studies in cells extremely difficult. For eYFP and DsRed ($R_{OPE} > 100$) single-molecule studies have been demonstrated [10]. This picture significantly changes for two-photon excitation. Indeed, due to the super-linear scaling of the two-photon absorption cross-section with the one-photon absorption cross-section (Table 1), the relative detection yield for all autofluorescent proteins becomes $R_{TPE} > 35$. Such high relative detection yield leads to the extremely low autofluorescent background compulsory for single-molecules studies in life cells. Preliminary data from our laboratory show that the autofluorescent background is indeed largely (by a factor of 10 or higher) reduced with respect to the autofluorescent background in one-photon measurements.

4. Conclusions

We have shown that the use of two-photon excitation does largely enhance the detection ratio of all autofluorescent proteins against the autofluorescence background expected from flavines in living cells. The higher detectability is the result of the super-linear scaling between the one- and two-photon absorption cross-sections which has been previously reported for organic fluorophores. The presentation of the two-photon spectra allows one to optimize the excitation wavelength for any particular experiment including those in which one strives to use fluorescence resonant energy transfer between the various autofluorescent proteins [8,9]. In conclusion, it should be mentioned that we have neglected the effect of photobleaching in our discussion, which is another factor important for single-molecule studies [10]. It has been generally found that the process of photobleaching is enhanced (typically by one order of magnitude) for two-photon excitation with respect to one-photon excitation [26]. This might expend the enhancement due to the favorable spectroscopic properties investigated here. Further studies of the photobleaching behavior dependence on the pulse width and wavelength, for example, will be needed to finally decide if two-photon excitation will be superior to one-photon excitation for single-molecule studies in cells.

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