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Two-photon absorption spectra of fluorescent isomorphic DNA base analogs

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Abstract: Fluorescent DNA base analogs and intrinsic fluorophores are gaining importance for multiphoton microscopy and imaging, however, their quantitative nonlinear excitation properties have been poorly documented. Here we present the two-photon absorption (2PA) spectra of 2-aminopurine (2AP), 7-methyl guanosine (7MG), isoxanthopterin (IXP), 6-methyl isoxanthopterin (6MI), as well as L-tryptophan (L-trp) and 3-methylindole (3MI) in aqueous solution and some organic solvents measured in the wavelength range 550 - 810 nm using femtosecond two-photon excited fluorescence (2PEF) and nonlinear transmission (NLT) methods. The peak 2PA cross section values range from 0.1 GM (1 GM = 10^{-50} cm^4 s photon^{-1}) for 2AP to 2.0 GM for IXP and 7MG. Assuming typical excitation conditions for a scanning 2PEF microscope, we estimate a maximum image frame rate of ~175 frames per second (FPS).

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Biomedical Optics EXPRESS

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1. Introduction

Two-photon excited fluorescence (2PEF), especially when combined with suitable fluorescent markers and wavelength-agile, high peak intensity ultrafast lasers, is a workhorse for studying biological matter. Two-photon absorption (2PA) spectra of extrinsic fluorescent dyes [1,2] and popular genetically engineered fluorescent proteins [3] have been reported extensively in the literature. In contrast, only limited data is available about the 2PA characteristics of DNA bases and amino acids, which are intrinsic to cells and tissues. This is mainly because the emission efficiency of natural fluorophores is often too low for practical use (quantum yields ~10^{-4} [4]). Highly fluorescent isomorphic analogs of DNA bases such as 2-aminopurine (2AP) and 6-methylosoxantropterin (6MI) offer potentially improved conditions for the 2PEF-based applications [4], provided that their 2PA is comprehensively characterized. Tryptophan (L-trp), whose relative 2PA spectral profile was reported already in the 1990s [5], is another promising intrinsic fluorophore for *in vivo* 2PEF studies [6–8], including imaging of single protein molecules [9]. Unfortunately, consistent data on the 2PA cross-section, σ_{2PA}, is still lacking [10–12]. In a study of leukocyte trafficking in cells, Li et al. [8] reported fast 2PEF imaging up to 30 frames per second (FPS) using intrinsic L-trp, however, significant uncertainty regarding the σ_{2PA} value hindered optimization of the technique.

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Here we determine the 2PA spectra and the peak σ_{2PA} values of 2AP, the ribonucleoside of 6MI (r6MI), 7-methyl guanosine (7MG, a methylated version of guanosine), isoxanthopterin (IXP, a guanine analog) and L-trp and its derivative 3-methylindole (3MI) in the 2PE wavelength range, 550 - 810 nm in different solvents including water, using three alternative methods based on the detection of relative- and absolute 2PEF signals and measuring relative nonlinear transmittance (NLT). Utilizing a previously derived relationship between σ_{2PA} and speed of multi-photon imaging [13], we estimate the maximum FPS that is achievable in a generic scanning 2PEF microscope. Furthermore, by applying a simplified model (so-called two-level model) for quantitative description of σ_{2PA} in a dipolar chromophore [14], we estimate the change of permanent electric dipole moment upon the transition from ground- to the lowest excited state.

2. Experimental

The relative 2PEF and NLT techniques were described previously in [15–17]. The absolute 2PEF method is described in [18]. Briefly, for relative measurements we used a regenerative amplified Ti:Sapphire femtosecond laser (Coherent Libra), operated at 1 kHz and 100 Hz pulse repetition rate for the 2PEF and NLT, respectively. The laser produced ~100 fs duration pulses at 800 nm with 2 mJ energy, which were used to pump the optical parametric amplifier (OPA) (Coherent OPerA Solo). The OPA wavelength was tuned in the region 550–810 nm in 1 nm steps. Depending on the wavelength, the OPA output had pulse duration 100 - 200 fs and the spectral width ~15 – 35 nm. 2PA spectral shapes were corrected in the range 550 - 720 nm relative to 9-chloroanthracene in dichloromethane (DCM) [15], in the 680-810 nm range relative to fluorescein in pH 11 aqueous buffer [18] and in the intermediate region 680-720 nm relative to an averaged of the above two standards. The σ_{2PA} values were measured relative to bis-diphenylaminostilbene (BDPAS) in DCM [18] for 6MI, while for all other samples 9-chloroanthracene in DCM was used as reference [15]. Relative quantum yields Q^R (relative to 9-chloroanthracene in DCM) were measured using a fluorometer (PerkinElmer LS50B). Absolute 2PEF measurements were performed for 6MI using a mode-locked Ti:Sapphire laser (Coherent Mira 900), where the wavelength was manually adjusted in the range 720 – 960 nm. The absolute σ_{2PA} was determined by calibrating the 2PEF signal relative to the linear excitation in the same sample and under the same detection conditions [18]. 2AP, 7MG, IXP, 3MI and L-trp were purchased from Sigma-Aldrich (>98% purity) and were used as received. r6MI with a ribose group at positon 8 was obtained from Fidelity Systems, Inc. (Gaithersburg, MD, USA) and was used as received. Distilled water was used to prepare aqueous solutions. Methanol (MeOH) and dimethyl sulfoxide (DMSO) were of HPLC grade and were used as received from Sigma-Aldrich. Glycerol-H_2O mixture (1:10) was used for 2AP. For 6MI and 7MG, a phosphate buffer (pH 7.03) was used. Due to low solubility of IXP in H_2O, we used NH_4OH-H_2O mixture (pH 11.5). Samples for the 2PEF and NLT experiments were contained in 1 or 10 cm path length quartz cuvettes, respectively. Concentrations of the solutions were determined using a spectrophotometer (PerkinElmer Lambda 950) and were calculated using published molar extinction values (Table 1).

3. Results and discussion

Figure 1 shows the 1PA (red lines) and 2PA spectra measured by the relative 2PEF method (empty black symbols) and the relative NLT method (solid blue symbols). The σ_{2PA} values obtained by the relative and absolute 2PEF techniques at select 2PA wavelengths, λ_{2PA}, are shown by green symbols. All three methods give consistent results, leading to estimated accuracy of the σ_{2PA} values ~35-40%. The highest peak σ_{2PA} value is obtained for IXP and 7MG, σ_{2PA} ~2.0 GM, while the lowest peak value is for 2AP in neat H_2O, σ_{2PA} = 0.12 GM (Table 1). Lane et al. reported for aqueous solution of 2AP, σ_{2PA} ~0.2 GM at λ_{2PA} = 584 nm using 2PEF method [19], which is larger than our result. Interestingly, the peak σ_{2PA} of 2AP varies substantially depending on the solvent, from 0.12 GM in neat H_2O and in neat MeOH

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to 0.2 GM in DMSO and Glycerol-H_2O mixture (Table 1). We note that two tautomeric forms of 2AP are present near neutral pH [19, 20]. Here we assume that a single species is dominant both in the linear and 2PA spectra. It would be beneficial to perform the NLT measurements and TD-DFT calculations for 2AP to confirm the obtained results and investigate in more details such effects that should be addressed in a future study. The 2PA shape of 2AP and 6MI follow closely the $S_0 \rightarrow S_1$ transition in the 1PA spectrum, independent of the solvent (Fig. 1(a)-1(d), 1(i)). Consequently, the optimal two-photon excitation wavelength in case of 2AP and 6MI in the chosen solvents is determined by the position of the maximum in the 1PA spectrum. This is consistent with an earlier report by Katilius et al. [22], where the 2PA spectrum of 6MI in H_2O (unfortunately pH was not provided) was measured in a narrower range, 700-780 nm. It was also reported that σ_{2PA} ~2.5 GM at λ_{2PA}, 700 nm [22], which is higher than our value, σ_{2PA} ~1.5 GM (Fig. 1(i)). In case of 7MG in pH 7 buffer, the long wavelength side of the 2PA and 1PA spectra show similar shape, but the 2PA maximum, σ_{2PA}~2 GM at λ_{2PA}~560 nm, appears to be slightly shifted

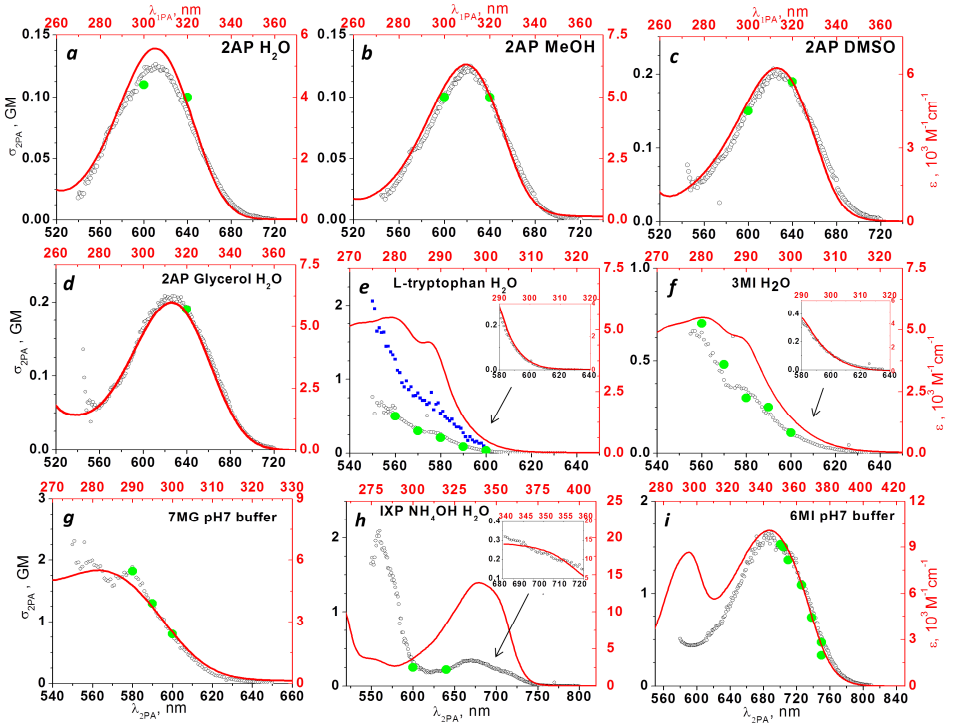


Fig. 1. 1PA and 2PA spectra of 2AP in H_2O (a), MeOH (b), DMSO (c), glycerol/H_2O (d), L-trp in H_2O (e), 3-MI in H_2O (f), 7MG (g) in pH 7 buffer, IXP in NH_4OH/H_2O (h), 6MI in pH 7 buffer (i). Left vertical axis - σ_{2PA} in GM. Right vertical axis - molar extinction, ε. The lower (upper) horizontal axis shows the 2-photon (1-photon) wavelength λ_{2PA} (λ_{1PA}) in nm. 1PA is shown by red lines; 2PA measured by the relative 2PEF method and the relative NLT method is shown by empty black symbols and solid blue symbols, respectively. σ_{2PA} obtained at select λ_{2PA} by 2PEF technique is shown by green symbols. Inserts in *d*, *e* and *g* show overlap between 1PA and 2PA spectral shapes.

towards shorter wavelengths (Fig. 1(g)). Interestingly, the 2PA spectrum of IXP (Fig. 1(h)) is quite distinct from the structurally closely related 6MI (Fig. 1(i)): it displays the maximum value, σ_{2PA} ~2 GM, at λ_{2PA} ~560 nm, whereas at the 1PA maximum position there is only a relatively weak peak with σ_{2PA} ~0.25 GM at λ_{2PA} ~670 nm. This observation may stem from the presence of different forms depending on pH [23]. L-trp (Fig. 1(e)) and its methylated derivative 3MI (Fig. 1(f)) show peak σ_{2PA} of 0.7 GM at λ_{2PA} ~550 nm and 0.6 GM at λ_{2PA}

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~560 nm, respectively. Previously, Meshalkin et al. found, σ_{2PA} ~0.16 GM at 530 nm [12] using 2PEF method, while other authors reported substantially smaller values, σ_{2PA} ~0.03 GM [11] and 0.005 GM [10] at 532 nm. Our 2PA spectral profiles (Fig. 1(e) and 1(f)) are similar to that reported in [5] and showed a long wavelength part that closely follows the 1PA profile (inserts in Fig. 1(e) and 1(f)). Also, near the 1PA peak at λ_{2PA} ~550-570 nm, the 2PA shape shows structure consistent with the indole dual excited electronic states model [5,24].

Expediency of using intrinsic fluorophores for 2PEF microscopy depends largely if they allow achieving fast frame rates at high spatial resolution. A recent quantitative model of 2PEF-based data acquisition in a diffraction-limited scanning beam microscope provides an estimate of the ultimate number of frames per second, FPS, achievable with given fluorophore characteristics [13]:

$$FPS \cong 1.24 \frac{8Ln(2)\cdot\eta\, C\, \sigma_{2PA}\, \lambda_{2PA}}{g\tau M_{FOV}\,(SNR)^2}\left(\frac{P_{av}}{\pi hc}\right)^2, \qquad (1)$$

where η is the combined fluorescence collection and detection efficiency, C is the fluorophore concentration (in m^{-3}), λ_{2PA} is the excitation laser wavelength (in m), g is the excitation source repetition rate (in Hz), τ is the excitation pulse duration (in s), M_{FOV} is the maximum pixel count in the microscope field of view (FOV), SNR is the minimum signal-to-noise ratio per pixel, P_{av} is the average power of the excitation (in W), h is the Planck constant (in J·s), c is

Table 1. Summary of 1PA and 2PA properties of the studied chromophore-solvent systems. C is the maximum molar concentration. Quantum yield Q^R was measured relative to 9-chloroanthracene in DCM at 450 nm. ε_M is the peak molar extinction coefficient. $\sigma_{2PA}^{2PEF}(\lambda_{2PA})$ and $\sigma_{2PA}^{NLT}(\lambda_{2PA})$ are the peak σ_{2PA} values at the 2PA wavelength (λ_{2PA}) measured by 2PEF and NLT methods, respectively. FPS values were estimated using Eq. (1) assuming fluorophore concentration 10^{22} m^{-3} (see text).

Comp.	Solvent	C	Q^R	ε_M	λ_{1PA}	$\sigma_{2PA}^{2PEF}(\lambda_{2PA})$	$\sigma_{2PA}^{NLT}(\lambda_{2PA})$	FPS	$\Delta\mu$
		mM		$M^{-1}cm^{-1}$	*nm*	*GM (nm)*	*GM*	s^{-1}	*D*
L-trp	H_2O	130	12	5500 [25]	279	0.7 (550)	1.5 (550)	100	2.1 ± 0.3
3MI	H_2O	5	2.3	5500 [25]	280	0.6 (550),	< 2.0 (550)	60	2.0 ± 0.3
7MG	pH 7 buffer	30	0.012	5500 [26]	258	1.8 (560)		150	2.0 + 0.5
IXP	NH_4OH H_2O	30	7	14000 [25]	340	2.0 (550)		175	1.0 ± 0.2
6MI	pH 7 buffer	10		10090	343	1.7 (680)		200	2.8 ± 0.2
2AP	H_2O	30	0.58	5560	305	0.1 (612)		10	1.1 ± 0.2
2AP	MeOH	30	0.8	6310	310	0.1 (622)		10	1.0 ± 0.1
2AP	DMSO	30	0.7	5930	313	0.2 (626)		15	1.2 ± 0.2
2AP	Glycerol H_2O	30	0.22	6250	312	0.2 (625)		15	1.1 ± 0.2

the speed of light (in m s^{-1}) and σ_{2PA} is in m^4 s $photon^{-1}$. The average concentration of L-trp in cellular structures is in the range, C ~10^{19} –10^{22} m^{-3} [27,28]. Assuming SNR = 10 and η = 0.15 [13], and evaluating other required parameters based on some published experiments such as [8], FOV~$(200\cdot10^{-6}$ m$)^2$ and minimum focus spot size, ~$2\cdot10^{-6}$ m (which gives M_{FOV} ~$9\cdot10^3$), g = 80 MHz, τ = 100fs, P_{av} = 10mW, we obtain that for e.g. IXP the highest achievable frame rate is 10-175 FPS (for other studied here molecules FPS values are shown in Table 1). Note that intrinsic and many isomorphic fluorophores such as IXP may be

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implemented at substantially higher concentration compared to e.g. fluorescent proteins without disrupting biological function. Under typical 2PEF microscope sample illumination conditions, the quantum efficiency of photobleaching of L-trp, φ~0.07-6.5% [9], is comparable or even lower than in fluorescent proteins (φ~0.9%) [29]. Therefore, even though the maximum peak σ_{2PA} of IXP may lack behind relative to some other types of probes, we estimate that the upper most FPS may still exceed what is typical for fluorescent proteins [13].

Finally, we take advantage of the observation that in the long wavelength part of the $S_0 \rightarrow S_1$ transition the 2PA spectra closely follows the corresponding 1PA shape, and evaluate the change of the permanent electric dipole moment, $\Delta\mu$. For typical dipolar molecules the absolute value of the latter quantity (in statC·cm) may be estimated from the relation [14]:

$$|\Delta\vec{\mu}| = \left(\frac{5}{12 \cdot 10^3 \, \pi \ln 10} \frac{\mathrm{nc}^2 h \mathrm{N_A}}{f^2} \frac{\sigma_{2PA}}{\varepsilon_M \, \lambda_{1PA}^{\max}} \right)^{1/2}, \qquad (2)$$

where ε_M is the peak molar extinction coefficient (cm^{-1} M^{-1}), $\lambda_{1PA}^{\max}$ is the peak 1PA transition wavelength (cm), n is the index of refraction of the solvent, $f = (n^2 + 2)/3$ is the optical local field factor, N_A is the Avogadro constant, c is speed of light (m s^{-1}), h is Plank constant (J·s). The corresponding $\Delta\mu$ values are collected in Table 1. In 2AP, where the peak σ_{2PA} showed a strong solvent dependence, $\Delta\mu$ is also varying in a broad range, 1.0 - 1.2 D (1D = 10^{-18} statC·cm) in all considered here solvents. Evans et al. used Stokes shift measurements to obtain $\Delta\mu$ ~2-5 D for 2AP in a variety of solvents including MeOH and DMSO [20], while Smagowicz et al. determined $\Delta\mu$ ~1.8 D [30]. For L-trp and 3MI in water we obtain, $\Delta\mu$ = 2.1 D and 2.0 D, respectively. Earlier, Pierce and Boxer used Stark spectroscopy [31] to study L-trp in glass matrix and reported for the lower-energy transition (L_α transition) a larger value, $\Delta\mu$ ~6 D, while Jalviste et al. [32] reported for the L_α transition in indole, $\Delta\mu$~5.6 D, and for 3MI $\Delta\mu$~7.2 D, both studied in polymethylmethacrylate films. It should noted that previous reports [31,32] provide $\Delta\mu$ without the local field enhancement factor, which may affect the $\Delta\mu$ values.

4. Conclusions

For first time we report on accurate measurements of the 2PA spectra and σ_{2PA} of a series of fluorescent isomorphic DNA base analogs as well as 3MI and L-trp in the 550-810 nm range. We verify our results by implementing three different experimental methods, thus alleviating inconsistencies in the earlier published literature. Based on our measured σ_{2PA} values, we estimated the maximum FPS achievable in a generic 2PEF microscope to be equal or even exceed that obtained with fluorescent protein under comparable conditions. We also estimated the change of permanent molecular electric dipole moment, $\Delta\mu$ = 1–3 D, which is in good agreement with values obtained previously using other methods. We anticipate that by providing reliable experimental 2PA spectra, this work will stimulate further development of applications that rely on multiphoton excitation of biological fluorophores.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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