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Applications of the time-resolved two-colour two-photon excitation of UV fluorophores using femtosecond laser pulses

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Abstract
A short overview of the principles and applications of the two-colour two-photon (2C2P) excitation of fluorescence by using femtosecond pulses is given. Fluorescence is generated by the simultaneous absorption of an 800 nm photon and a 400 nm photon of overlapping laser beams of a titanium:sapphire laser. Two examples of its application are presented: firstly, it is used to monitor the enzymatic cleavage of bovine serum albumin (BSA) by elastase. The fluorescent amino acid tryptophan present in BSA is excited corresponding to an effective one-photon wavelength of 266 nm. Secondly, it is shown how one can utilize the different polarizations of the excited beams for determining the symmetry of the excited states of molecules, exemplarily shown for p-terphenyl in cyclohexane. Further applications and experiments for 2C2P are suggested for using it in UV-fluorescence microscopy and for determining the properties of the electronic states of biomolecules by using differently polarized photons.

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(Some figures in this article are in colour only in the electronic version.)

1. Introduction

Fluorescence studies have a long history in molecular spectroscopy. Detecting fluorescence instead of absorption not only allows bringing sensitivity to a higher level but also gives additional information about the species under observation since the fluorescence of a molecule is strongly influenced by its surroundings.

Especially in the biosciences, fluorescence studies, i.e. microscopy, have become an indispensable tool offering localization of protein expression [1] and selective monitoring of intracellular parameters like pH [2] or calcium concentration [3] by introducing fluorescent labels into cells and organisms [4]. Multi-photon excitation of fluorescence has long been used in molecular spectroscopy [5], but it was the invention of the two-photon microscope (TPM) in the early 1990s that introduced this technique to fluorescence microscopy [6]. It is superior for deep tissue imaging as well as for high-speed *in vivo* imaging [7]. The key to its success is the possibility of providing detailed and minimal invasive insight into the function of biological systems under genuine conditions. Compared to standard fluorescence microscopes that excite fluorescence via one-photon absorption processes, two-photon microscopy provides a number of advantages arising directly from the two-photon absorption process, e.g. intrinsic 3D resolution and limitation of photo damage to the focal region.

Nowadays, a source for the two-photon excitation of a wide range of fluorophores with TPM is the titanium:sapphire (Ti:Sa) laser. It is highly efficient for multi-photon excitation because of its stable performance, short pulses and high repetition rate, but its spectral window is limited to roughly

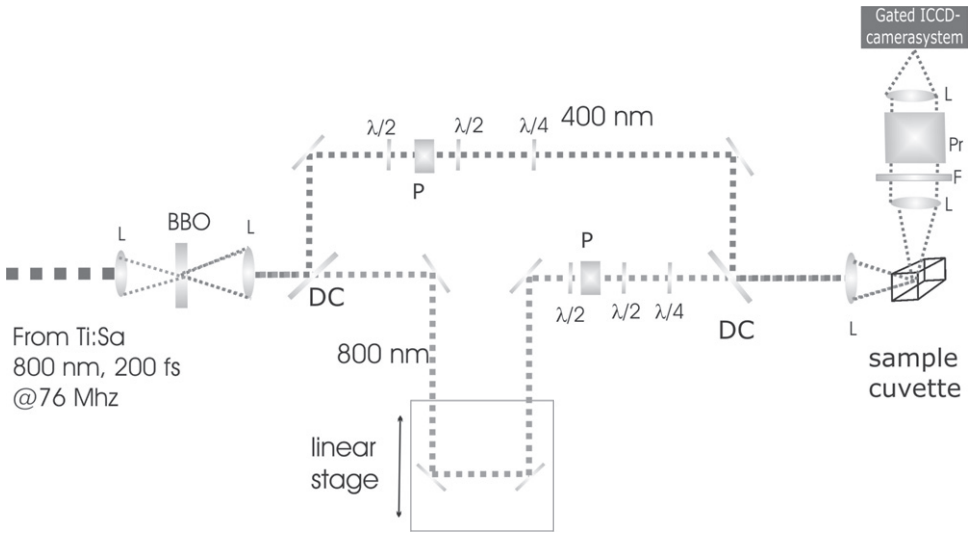

Figure 1. Experimental setup for 2C2P excitation: DC, dichroic mirror; P, thin layer polarisator; L, lenses; BBO, beta-barium-borate crystal; λ/2–λ/4, half/quarter-wave plate; F, a series of filters; Pr, Rochon prism.

700–1000 nm, e.g. 350–500 nm for one-photon excitation. Hence, efficient excitation of ultraviolet (UV) fluorophores such as tryptophan, absorbing mainly between 230 and 300 nm, is not possible. But especially for UV fluorophores, multi-photon excitation would be desirable. One reason for this is that applying UV light to a biological sample for one-photon excitation of fluorescence will inevitably result in high photo damage [8]. All nucleic bases absorb strongly in similar spectral regions, an effect that is used e.g. for sterilizing equipment in biological research.

Since no femtosecond laser light at 560 nm is easily available, performing a two-colour two-photon (2C2P) excitation is a way of overcoming this problem, i.e. two photons of different colours are used for a two-photon excitation. 2C2P excitation in the UV has been shown before by Lakowicz for UV fluorophores with a picosecond laser [9–11]. Recently, we demonstrated the first realization of the 2C2P excitation using femtosecond laser pulses for a range of UV laser dyes [12]. Excitation with femtosecond pulses can provide superior time resolution in time-resolved measurements and higher signal-to-noise ratio without damaging the sample.

In this paper, we review two applications of 2C2P excitation in the UV with time-resolved measurements. They show the wide range of useful applications for this technique. The first is related closely to the biosciences and molecular screening, whereas the second is related to multi-photon spectroscopy.

The fluorescent amino acid tryptophan is present in many proteins and its absorption maximum is at 280 nm; therefore, it can easily be excited with 2C2P using an 800 nm photon and a 400 nm photon resulting in an effective excitation wavelength of 266 nm. Since the fluorescence lifetime of tryptophan is extremely sensitive to its surroundings, it can provide information about the protein itself and about its reactions [13, 14]. As a first application, we use this environmental sensitivity for monitoring the fast enzymatic cleavage of the protein bovine serum albumin (BSA) by elastase. A similar slower enzymatic reaction using the same technique has been described previously [15].

Secondly, we make use of the polarization properties of the excitation beams. Single-colour multi-photon techniques have been used to measure polarized two-photon spectra to elucidate the nature of the excited state of UV molecules in solution [16, 17]. There are different selection rules for one- and two-photon spectroscopy; hence, one-photon 'forbidden states' can be characterized. The two-photon absorption is polarization dependent, and by using differently polarized photons (circular corotating/conrotating and linear parallel/perpendicular), the symmetry of the excited state can be assigned. The theory of this dependence was described by McClain [18] and has been extended to two-photon-induced fluorescence [19, 20]. A number of independent molecular parameters that describe the symmetry and the orientations of the two-photon tensor and transition moment of the fluorescence can be determined by conducting a set of fluorescence polarization experiments.

However, a complete set of molecular parameters for fluorophores in a liquid can only be obtained by exciting with photons of different colours, as shown by McClain and Wirth [5, 21], because the experiments have to involve photons of different wavelengths and different polarizations. Wan and Johnson showed that a highly time-resolved analysis is needed as well [22]. Additionally, certain excited states can only be observed with a two-colour setup as they are formally forbidden for single-colour two-photon excitation [21]. To the best of our knowledge, such complete experiments have not been performed so far. We present the first results for the laser dye p-terphenyl (PTP) in cylcohexane, which indicate that measurement of the parameters is possible with 2C2P excitation using femtosecond pulses.

2. Experimental setup and results

The general setup for 2C2P excitation is shown in figure 1. The fundamental output of a Ti:Sa laser is focused into a

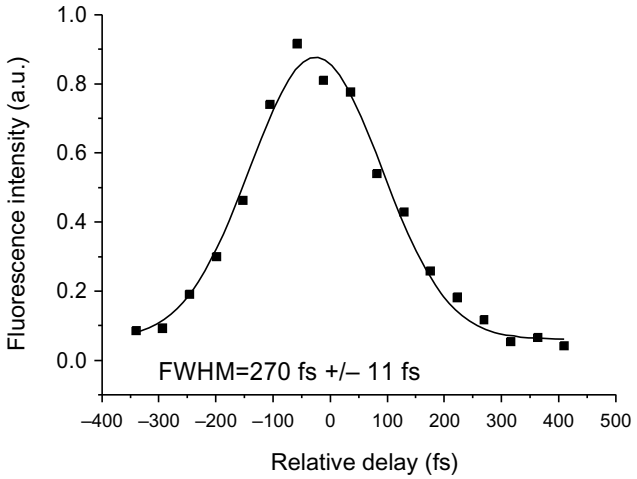

Figure 2. Cross-correlation experiment with a 0.1 mM solution of BSA in PBS; relative delay between the 400 and 800 nm pulses, full width at half maximum (FWHM) 270 fs; taken from [15].

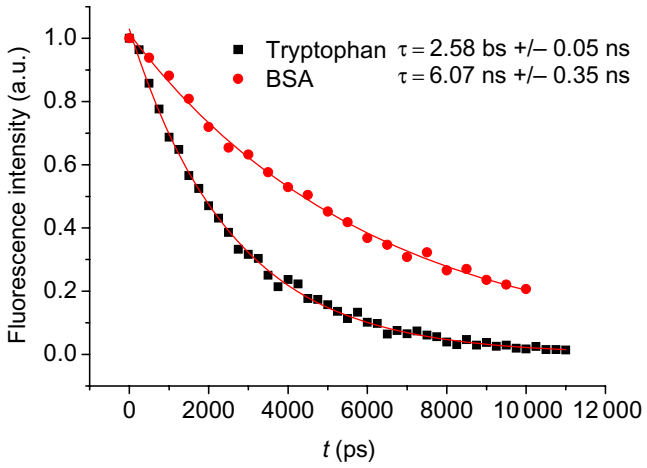

Figure 3. Fluorescence intensity decay curves of 0.1 mM solution of BSA and tryptophan in PBS; lifetime values of 2.58 ns (tryptophan) and 6.07 ns (BSA) were obtained by a single exponential fit.

BBO crystal for frequency doubling. The 800 and 400 nm beams are separated by a dichroic mirror (DC). The intensity of each beam can be controlled by a $\lambda/2$ and a thin layer polarizator. The polarizations can additionally be controlled by half- and quarter-wave plates and the path length of the 800 nm beam can be adjusted by a linear stage/delay line. The beams are united by a second dichroic mirror and focused into the sample cuvette. The fluorescence is detected by a gated intensified charge-coupled device (ICCD) camera system. It allows us to measure fluorescence intensity decays. A Rochon prism is used to analyse the polarization of the fluorescence, e.g. for anisotropy decays. The experimental details have been described previously [15].

In contrast to single-colour multi-photon techniques, where the two absorbed photons are already synchronized, both photons have to be spatially and temporally aligned for simultaneous absorption. In addition one should control the power, the path length and the polarization of both colours. Both photons have to be spatially and temporally aligned for simultaneous absorption.

The importance of the delay line/linear stage is demonstrated in figure 2 with a cross-correlation experiment. The highest 2C2P intensity signal is achieved when both photons are perfectly temporally aligned. The FWHM of the cross-correlation curve reflects the pulse width of the 800 nm beam.

Analogous to single-colour two-photon (1C2P) excitation, where the fluorescence intensity depends on the square of the power of the laser beam, the two-photon absorption strength, the fluorophore concentration, etc, the intensity of the 2C2P fluorescence depends on the product of the power of each beam. The dependence on the product is of experimental advantage because with an excess of the 800 nm light and only a small amount of the 400 nm light, one can still retain the same fluorescence intensity, thus reducing the background 1C2P-excited fluorescence by the 400 nm beam and reducing the photo damage.

As mentioned above, one of the first applications of 2C2P fluorescence was the label-free monitoring of a proteolytic digestion of the protein BSA by subtilisin [15]. BSA contains five tryptophans, which act as a natural built-in probe for their surroundings. The fluorescence intensity decays for BSA and tryptophan measured by 2C2P excitation of fluorescence are shown in figure 3. The fluorescence lifetimes were obtained by applying a single exponential fit to the data and are in good agreement with values from the literature [23, 24].

In addition to the above-mentioned proteolytic cleavage of BSA by subtilisin, a similar reaction between BSA and elastase is monitored. The experimental setup is shown in figure 1 and was described in detail previously [15]. The reaction is performed at concentrations of 0.1 mM BSA and 0.01 mM elastase. The fluorescence intensity decay and the decay of the fluorescence lifetime during the reaction are shown in figures 4(A) and (B). Similar to the digestion with subtilisin, the fluorescence lifetime decreases during the enzymatic reaction. The rate of the reaction is relatively high. After 5 min, the fluorescence lifetime has dropped from 6 to 4.3 ns and does not decrease further significantly.

The reaction of BSA with elastase is far too fast to be monitored by gel electrophoresis parallel to the fluorescence lifetime measurements. Figure 5 shows a photograph of the electrophoresis run after 30 min of reaction time. Gels run after a longer period of reaction time still showed a BSA residue (at 66 kDa) indicating that elastase is not able to digest BSA completely. After reaction with elastase, fragments with higher molecular weights are predominant compared to those found after reaction with subtilisin. Apart from the band at E, which can be assigned to elastase (molecular weight of 27 kDa), the majority of fragments have molecular weights of about 35 kDa and some below 30 kDa.

To ensure that the measurements of the fluorescence lifetime are not significantly influenced by anisotropic effects, the fluorescence anisotropy decay of BSA was measured (data not shown). The initial anisotropy is as low as 0.07. This very low anisotropy does not allow a determination of the rotational diffusion constant with an acceptable accuracy. However, it clearly demonstrates that the fluorescence lifetime measurements are not disturbed by anisotropical effects.

The change of the lifetime clearly shows the progression and the end of the protein reaction. The lifetime after 60 min

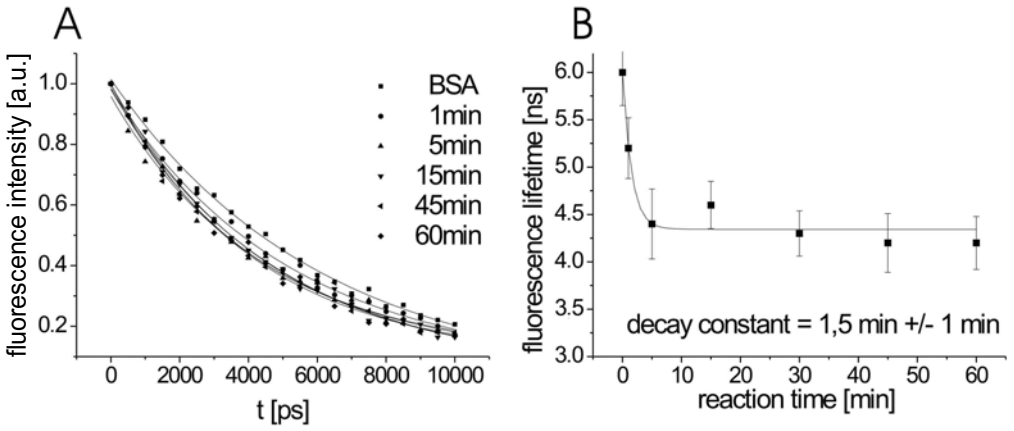

Figure 4. (A) Fluorescence intensity decays at different times of the enzymatic reaction of BSA and elastase. (B) Decay of fluorescence lifetime during the reaction. The acquisition time for a single lifetime curve was less than 1 min.

Figure 5. Gel electrophoresis of the reaction mixture of 0.1 mM BSA and 0.01 mM elastase quenched at different times of the reaction. Molecular weights of the marker proteins: (A) 66 kD, (B) 45 kD, (C) 36 kD, (D) 29 kD, (E) 24 kD, (F) 20.1 kD, (G) 14.2 kD, (H) 6.5 kD.

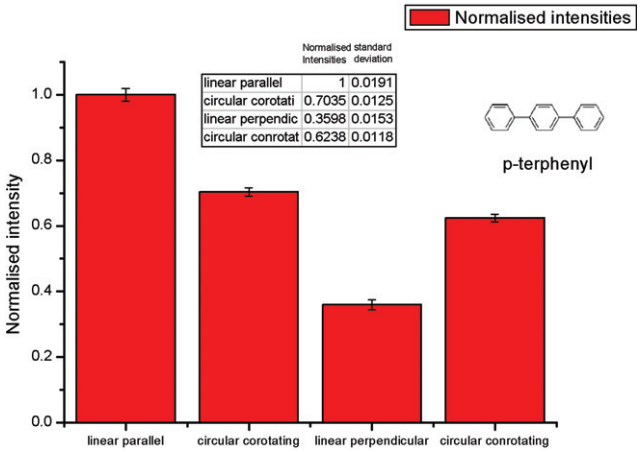

Figure 6. 2C2P experiment using differently polarized photons (400 and 800 nm) (fluorophore: 1 mM PTP in cyclohexane), and normalized fluorescence intensities I_{par}, I_{per}, $I_{circ.co}$ and $I_{circ.con}$.

is much higher than the lifetime of the free tryptophan, which indicates that the tryptophans are still in a protein analogue environment. This is in agreement with the electrophoresis data.

Another application of 2C2P excitation is molecular spectroscopy. As noted in the introduction, the two-photon absorption is polarization dependent. The relative two-photon absorption can be measured by detecting the total fluorescence intensity at different excitation polarizations. The laser dye PTP in cyclohexane was excited using the setup described above without the Rochon prism. Previous experiments have shown (data not shown) that the emission is completely isotropic about 600 ps after the pulse; therefore, the fluorescence intensity was detected at that time in order to exclude anisotropic contributions. The polarizations were changed using the wave plates, while keeping the intensities of the beam excitations constant for each case. The effect of using differently polarized photons is shown in figure 6. The maximum intensity was found for parallel polarization (I_{par}); the lowest was obtained for perpendicular polarization (I_{per}). The signal intensities at different polarizations are not totally independent of each other. Knowing three intensities, the fourth can be predicted by using the equation [25]

$$I_{circ.co} + I_{circ.con} = I_{par} + I_{per}, \tag{1}$$

where $I_{circ.co}$ (circular corotating) is the observed intensity with both the excitation photons circular-polarized in the same direction (either right handed or left handed), whereas $I_{circ.con}$ (circular conrotating) is the observed intensity for oppositely circular-polarized excitation photons. I_{per} and $I_{circ.con}$ can only be measured with two laser beams of different polarization, like in our 2C2P setup. The obtained normalized intensities shown in figure 6 are in agreement with equation (1). Another important value in two-photon spectroscopy is the two-photon polarization ratio $\Omega = I_{circ.co}/I_{par}$. The variable Ω can have values from 0 to 3/2 [20] and has been used many times for the assignment of excited states [16, 25]. The maximum value of 3/2 is observed for a transition from the ground state to a non-totally symmetric excited state (e.g. $B_g \leftarrow A_g$). All other values indicate a transition from a ground state to a totally symmetric excited state (e.g. $A_g \leftarrow A_g$). Therefore our obtained value of 0.70 indicates that the excited state is totally symmetric.

It was shown by McClain [19] that seven molecular parameters can be determined by conducting

a 2C2P fluorescence experiment: three absorption parameters δ_F, δ_G and δ_H that allow further symmetry assignment of the excited state and the two-photon tensor pattern; and four other parameters that characterize the anisotropy of the fluorescence. δ_F is the absolute square of the trace of the (3×3) two-photon tensor S_{ij}, δ_G is the sum of the absolute squares of all the tensor elements and δ_H is the sum of the products of the tensor elements times the complex conjugate of its transpose. The measured two-photon absorptivity (in our case the measured total fluorescence intensity) depends on these three absorption parameters and the polarization of the exciting photons.

By measuring I_{par}, I_{per}, $I_{circ.co}$ and $I_{circ.con.}$, the relative values of δ_F, δ_G and δ_H can be calculated [25]: δ_F is 1.55 ± 0.07, δ_G is 1.69 ± 0.08 and δ_H is 1.70 ± 0.08. Using the tables for the assignment of excited states [18], a value for δ_F that is greater than zero is an indicator for a totally symmetric excited state, while almost equal values for δ_G and δ_H indicate that the two-photon tensor elements $S_{ij} = S_{ji}$, i.e. the two-photon tensor is symmetric.

Compared with the setup used by Drucker [16] with its ruby laser and xenon flash lamp, the presented setup is less complicated, and using the Rochon prism it also offers polarized time-resolved measurements. Therefore it can be used to determine all relevant molecular parameters.

3. Conclusion and further directions

We have shown two applications for time-resolved 2C2P excitation using femtosecond laser pulses of a single Ti:Sa. It has been used to monitor the progression of a fast enzymatic reaction without any extrinsic labelling by changes of the fluorescence lifetime. Its advantages include the strongly reduced photo damage to the sample when compared to one-photon excitation and the higher time resolution when compared to gel electrophoresis. Strong fluorescence signal allows for short acquisition times of the fluorescence lifetime curves. This technique can also be applied to other protein reactions. Extending this 2C2P setup to a microscopic setup can easily be achieved, thereby offering smaller sample volumes and stronger fluorescence signals with the use of high-numerical-aperture objectives. With a 2C2P microscope, the imaging of intrinsic protein fluorescence in living cells is feasible.

We have also shown that this technique is useful in molecular spectroscopy. The theoretical groundwork describing the simultaneous absorption of two photons followed by emission has been laid in [19]. Using differently polarized photons, different absorbtivities can be measured for the assignment of the excited state, which was shown for PTP. This is the first step in performing a complete set of fluorescence polarization experiments. Probably due to a lack of stable laser sources and experimental difficulties, a large number of fluorescence polarization experiments have not been performed so far. With stable femtosecond laser sources, the availability of ultrafast photomultipliers and reliable time-resolved techniques such as time correlated single photon counting (TCSPC) and 2C2P, the viability of these polarization experiments is at hand.

The advantages of multi-photon excitation fluorescence microscopy will not only be retained but exceeded [26] when the additional technical possibilities of manipulation of the two beams at different wavelengths, intensities and polarizations are considered. Hence, 2C2P will provide us with a spectroscopic imaging technique possessing high spatial resolution, low photo damage and the advantage of a newly gained spectral region, which can be used for label-free detection of proteins. Additionally, the pulsed excitation offers a good variety of different fluorescence analysis techniques such as fluorescence lifetime imaging (FLIM) and time-resolved fluorescence anisotropy imaging (tr-FAIM) [27].

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