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ORIGINAL PAPER

Two-Color Two-Photon Fluorescence Laser Scanning Microscopy

S. Quentmeier · S. Denicke · K.-H. Gericke

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Abstract We present the first realization of a Two-Color Two-Photon Laser-Scanning Microscope (2c2pLSM) and UV fluorescence images of cells acquired with this technique. Fluorescence is induced by two-color two-photon absorption using the fundamental and the second harmonic of a Ti:Sa femtosecond laser. Simultaneous absorption of an 800 nm photon and a 400 nm photon energetically corresponds to one-photon absorption at 266 nm. This technique for Laser-Scanning Microscopy extends the excitation wavelength range of a Ti:Sa powered fluorescence microscope to the UV. In addition to the known advantages of multi-photon microscopy like intrinsic 3D resolution, reduced photo damage and high penetration depth 2c2pLSM offers the possibility of using standard high numeric aperture objectives for UV fluorescence imaging. The effective excitation wavelength of 266 nm corresponds especially well to the excitation spectrum of tryptophan. Hence, it is an ideal tool for label free fluorescence studies and imaging of intrinsic protein fluorescence which originates mainly from tryptophan. Thus a very sensitive natural lifetime probe can be used for monitoring protein reactions or changes in conformation. First measurements of living MIN-6 cells reveal differences between the UV fluorescence lifetimes of the nucleus and cytoplasm. The significance of this method was further demonstrated by monitoring the binding of biotin to avidin.

S. Quentmeier (✉) · S. Denicke · K.-H. Gericke
Institute for Physical und Theoretical Chemistry,
University of Braunschweig,
Hans-Sommer-Str. 10,
38106 Braunschweig, Germany
e-mail: s.quentmeier@tu-bs.de

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Introduction

In the field of fluorescence microscopy two-photon microscopy (2PM) has become an indispensable tool over the last twenty years [1]. Its sharp and high contrast pictures combined with its intrinsic 3D resolution and a superior penetration depth into biological tissue make it the technique of choice for many applications. Especially in the growing field of medical applications of fluorescence microscopy it is the reduced phototoxicity and high penetration depth that makes it the favorable technique [2–4].

But even though it is used in a huge variety of different applications it is still limited to a rather small spectral window. Today, the light source of choice for 2PM is still the Ti:Sa femtosecond laser providing an output of about 700 nm to 1,000 nm. Hence, the effective two-photon excitation is limited to a wavelengths range of 350 nm to 500 nm. In principle, an additional spectral window can be reached by using three-photon excitation [5]. However, due to extremely small three-photon absorption cross sections the power levels that have to be used are in most cases not suitable for biological samples.

One way to overcome these limitations is to extend two-photon excitation to two-color two-photon (2c2p) excitation [6]. Basic experiments and theoretical considerations towards two-color two-photon laser scanning microscopy (2c2pLSM) have been published earlier [7–13]. We demonstrate the practicability of two-color two-photon laser scanning microscopy in an experimental setup using the fundamental output of a Ti:Sa at 800 nm and the second

harmonic at 400 nm. By spatially and temporarily overlapping these beams two-color two-photon absorption with an effective excitation wavelength of 266 nm can be achieved. Hence, with 2c2pLSM the excitation range of the microscope is extended to the UV, i.e. 230–330 nm.

This spectral window is of special interest for life sciences, because it corresponds well with the absorption spectrum of tryptophan. Tryptophan is the amino acid which is responsible for the majority of intrinsic protein fluorescence [14]. Which does not only allow label free protein detection but also monitoring of protein reactions and conformational changes in proteins. This is possible because tryptophan represents a natural built-in probe whose fluorescence lifetime is sensitive chemical or conformational changes in its environment [15, 16].

The most obvious way to excite tryptophan is of course a one-photon excitation using 280 nm light. However, using this wavelength for excitation in confocal or widefield fluorescence microscopy creates numerous problems. First of all, UV light is toxic to living cells, especially because DNA also absorbs at these wavelengths [17]. Secondly, the shorter the wavelength becomes the more scattering occurs in turbid media resulting in dramatically reduced spatial resolution [18]. And last, such wavelengths require special optics which are not available nowadays. None of the high numeric aperture objectives ideally used for fluorescence microscopy can be used as they are not transparent for wavelengths shorter than 300 nm. Special UV objectives are available, but only with poor numerical apertures. Hence, they offer only low photon efficiency and low resolution.

2c2pLSM can be an solution to these problems [9, 11]. Using 800 nm and 400 nm for excitation of intrinsic protein fluorescence limits the absorption and photo damage to the focal region as known from other multi-photon techniques. Thus, compared to one-photon excitation where excitation occurs in a vast volume of the sample multi-photon techniques reduce the photo damage by exciting only where fluorescence is desired for detection. Furthermore, the employment of longer wavelengths reduces scattering.

Using 800 nm and 400 nm light for excitation conventional high numeric objectives can be used for 2c2pLSM, providing the best basis for a high resolution. Due to the Stokes shift the fluorescence occurs at wavelengths longer than 300 nm where the transmission efficiency is significantly higher.

The same high penetration abilities of the 800 nm light apply as in conventional 2PM keeping the photo damage low and the resolution high, because the 2c2p absorption probability depends on the product of the powers of each color. This allows increasing the excitation power at 800 nm light and accordingly reducing the power at 400 nm without loss of signal. Thus, the increased scattering and photo toxicity of the 400 nm light is of minor importance since only very low powers of it are required in the focus. The resolution is predominantly determined by the focus of the 800 nm light and its superior focusing properties in turbid media [19]. Light at 532 nm would be more strongly scattered than light at 800 nm. Thus, a lower resolution could be expected for a one-color two-photon excitation experiment.

Using a non-descanned detection in combination with sample scanning we present the first 2c2pLSM-fluorescence images of MIN 6 cells demonstrating the applicability of this method for UV-fluorophore imaging inside living cells. Additionally, the application of 2c2pLSM for monitoring the binding of biotin to avidin is demonstrated using a time-gated camera combined with galvo-optic beam scanning.

Experimental setup

Two different schemes of the experimental 2c2pLSM setup are shown in Fig. 1. The initial experimental setup is shown featuring a beam scanning setup with a time gated intensified charge coupled device (ICCD) camera (Fig. 1, B). To enhance photon efficiency and spatial resolution we switched to a non-descanned sample scanning setup with time correlated single photon counting (TCSPC) detection (Fig. 1, A). The basic setup in front of the microscope is the same for both setups.

The fundamental of a $NdYVO_4$ laser pumped Ti:Sa laser at 800 nm is frequency doubled by a beta-bariumborate (BBO) crystal as shown in Fig. 1. The remaining 800 nm light and the frequency doubled light are first separated by a dichroic mirror (DC_1) and reunified in the same way later (DC_2). Along their separated light paths the power and polarization can be individually adjusted. Additionally, the optical path length of the 800 nm beam can be matched to that of the 400 nm beam by an optical delay line consisting of two mirrors mounted on a motorized linear stage, ensuring temporal overlap of both laser pulses in the focal volume of the objective. Furthermore, the blue light path contains a telescope (T_1) which can be adjusted to account for chromatic aberrations relative to the 800 nm light in the subsequent optical pathway. After reunion both colors pass a telescope (T_2) for the purpose of beam expansion. The center of the galvo scanner is then projected by a third telescope (T_3) via a dichroic mirror DC_3 onto the back aperture of the objective (Zeiss plan-Neofluar 40x/1.3 oil) to ensure optimal scanning process in case of beam scanning (Fig. 1, B) using a galvo scanner (GSI Lumonics).

Fluorescence is collected by the same objective and transmitted by the dichcroic mirroc DC_3. After further isolation of the fluorescence by a set of filters F, the fluorescence image is projected by a quartz lens onto the

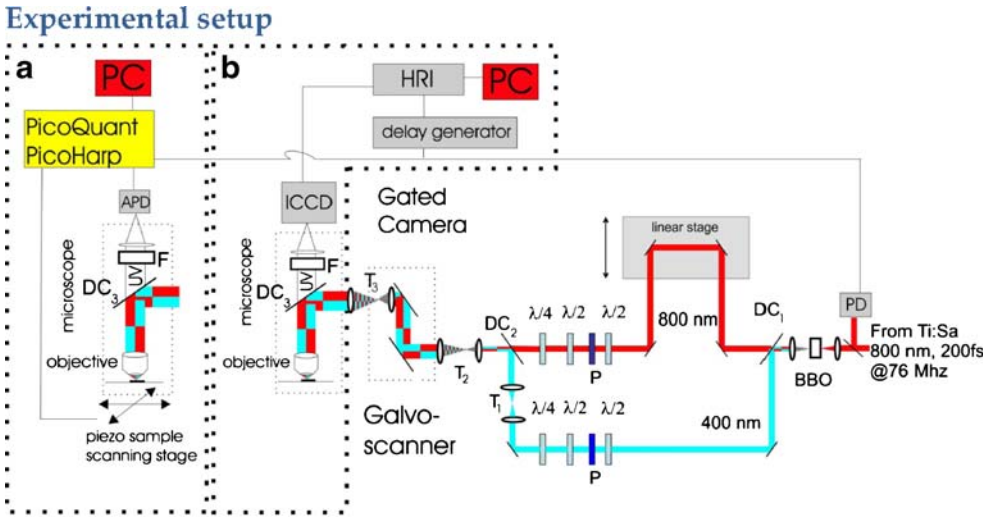

Fig. 1 Experimental Setup of a 2c2pLSM using either the setup *A* or *B*. *A* represents the setup with avalanche photo diodes (APD) and TCSPC detection and sample scanning while *B* shows the setup with time gated camera system and beam scanning. With *PD* photo diode; *BBO* beta-bariumborate crystal; *DC* dichroic mirror; λ/2 half wave plate; λ/4 quarter wave plate; *P* polarizer; *F* set of filters: interference filter 350/40 nm, notch filter 405 nm; *ICCD* intensified charge coupled device; *APD* avalanche photo diode; *TCSPC* time correlated single photon counting

photo cathode of a time gated ICCD camera system (LaVision GmbH, Göttingen, picostar). Alternatively, the camera system can be exchanged by an avalanche photo diode (APD, FWHM of IRF 250 ps at 400 nm) (micro photonic devices, Bolzano, PDM series) in combination with a TCSPC system (PicoQuant, Berlin, PicoHarp 300). Scanning is then achieved using a piezo sample scanner (Physik Instrumente GmbH Karlsruhe, P-733.2CL), with the galvo mirrors set to a defined zero position.

For initial alignment of the two laser beams the camera detection shown in Fig. 1, B is used. Therefore, both laser beams are coupled into the microscope and focused through the objective into a solution of p-terphenyl (PTP) in cyclohexane. At elevated laser power fluorescence can be detected from both foci. Previous investigations have shown that this fluorescence originates from a one-color two-photon excitation in case of the 400 nm beam and from a three-photon excitation in case of the 800 nm beam [6]. The fluorescence wavelength is around 350 nm. After spatial overlap is ensured temporal overlap is achieved by adjusting the optical path length of the 800 nm beam with the linear stage. Temporal and spatial overlap results in strongly increased fluorescence signal originating from the focus of the two aligned laser beams. Finally, the power of both beams can be reduced to a level, where only minor one-color two-photon excitation and almost no three-photon excitation occur. Due to the quadratic and cubic power dependence of these excitations they decrease more strongly than the 2c2p signal which is only linearly dependent on the power of each beam. Hence, 1c2p excitation with 400 nm light has a negligible contribution to the 2c2p image.

MIN 6 cells are cultivated in DMEM high glucose (4.5 g/L) (PAA laboratory GmbH) under carbogene atmosphere. Avidin and biotin are purchased from Sigma-Aldrich and used without further purification.

Results

Using the camera setup (Fig. 1, B) a cross correlation experiment was performed demonstrating that the total fluorescence measured originates from a 2c2p excitation. The delay of the red beam was successively varied by moving the linear stage. Figure 2 presents the resulting data. The solid line represents a Gaussian function fitting the data best with a full width at half maximum (FWHM) of 545 fs.

Strong fluorescence is only present when both laser pulses temporally overlap in the focal volume. Each color alone gives only a weak fluorescence signal, although increase of the excitation powers leads to fluorescence upon exclusive irradiation of each color. All subsequent measurements are performed at power levels where the background signal from irradiation with one color only is reduced to negligible level of percent. The absolute power applied to the sample for acquisition of the images shown was 15 mW at 800 nm and 0.4 mW at 400 nm.

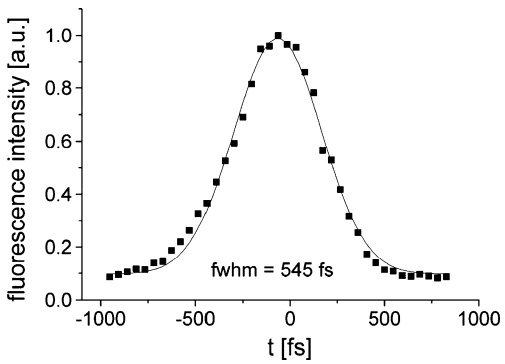

Fig. 2 Cross correlation experiment where the 800 nm pulse was delayed with respect to the 400 nm beam. The solid curve represents a Gaussian function fitting the data best

For cell imaging the non-descanned detection method in combination with the sample scanner is used. Figure 3 shows a UV fluorescence image of a MIN 6 cell acquired with this setup. Fluorescence intensity is detected at 350 nm with the APD TCSPC system and integrated over all relevant time channels. In the lower quarter of this image two black stripes can be seen. In these areas one of the two excitation laser beams was blocked. The black stripes prove that practically no fluorescence was observed by one-color irradiation.

The average fluorescence decay data collected over the designated areas is shown in the diagrams to the right in Fig. 3. Summation over the pixels in the designated areas renders two fluorescence decay curves that are of good quality as they show only little noise. A biexponential decay was found to fit the data best. The fluorescence lifetimes in the cytoplasm are significantly longer than those found in the nucleus.

An example for the significance of fluorescence lifetime measurements of intrinsic protein fluorescence is shown in Fig. 4. Using the camera beam scanning setup as described above (Fig. 1, B) the binding of biotin to avidin is monitored. The data represent the fluorescence decay data of a 1 mM solution of avidin in PBS and the data collected after addition of an equimolar amount of biotin. The data were integrated over a scanning area of about 10 µm×10 µm. A biexponential decay was found to fit the data best. Adding more biotin does not reduce the fluorescence lifetime further. We performed a global least square fit with shared decay parameters. Two mean lifetimes, 0.5 ns +/- 0.02 ns for the short and 2.1 ns +/- 0.08 ns for the long lifetime component are determined with good accuracy. For avidin a ratio of the preexponential factors of the short and the long lifetime of approximately 1:3 was found. For avidin in the presence of equimolar biotin a ratio of almost 3:1 was found.

Discussion

With Figs. 3 and 4 we present first applications of the concept of 2c2pLSM. The applicability of this concept is demonstrated by imaging UV fluorophores inside living cells as well as by monitoring of the biotin binding to avidin. Our results show that the fluorescence intensity generated by 2c2p excitation is sufficient for label free cell imaging and monitoring changes in intrinsic protein fluorescence lifetimes.

The cross correlation experiment performed with the microscope setup using a Zeiss plan-Neofluar 40x/1.3 oil immersion objective renders a cross correlation curve with a FWHM value of 545 fs. In previous experiments a FWHM value of 270 fs was found. This corresponds to a pulse broadening by a factor of 2 [6]. The main difference between the two setups is that the former contained no lenses except one achromatic lens for focusing the beams into the sample cuvette while in the present microscope setup there are five additional lenses and one microscope objective. These optical elements are responsible for the dispersive broadening of the pulses and, hence, the cross correlation curve. Therefore a major improvement can be achieved by implementing a pre-chirp setup to compensate for the dispersion effects. When the pulse length of each beam is thereby reduced to its original value an up to four

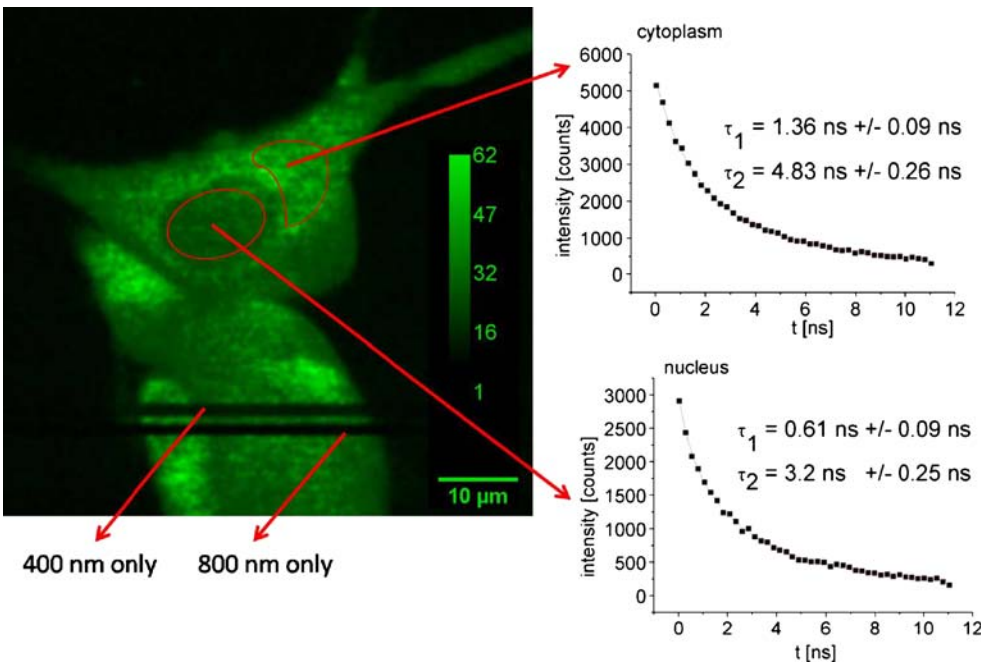

Fig. 3 2c2pLSM image of MIN6 cells. Graphs on the right show fluorescence decay data averaged over the marked areas. The solid lines represent a biexponential function which fits the data best. τ_1 and τ_2 are the fluorescence lifetimes corresponding to this fit. Fluorescence lifetimes in the cytoplasm are higher than in the nucleus. The dark stripes (400 nm only, 800 nm only) show that other processes than 2c2p absorption are negligible

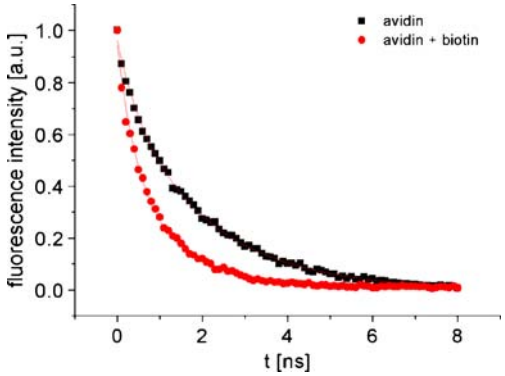

Fig. 4 2c2p excitation of avidin fluorescence. Intrinsic fluorescence lifetime of a 1 mM avidin solution in PBS (*black*) and a solution containing 1 mM avidin and 1 mM biotin in PBS (*red*). Displayed data represent mean values of 3 measurements for avidin and avidin plus biotin, respectively. Solid lines represent a global biexponential decay with shared decay parameters fitting the data best. Parameters are shown in Table 1

times higher excitation efficiency can be expected. However, with this 2c2p setup, an experimental setup is available for the first time for which the pulse broadening by the objective can be measured easily.

For 2c2p imaging the intensity of each laser beam is kept so low that almost no fluorescence is detected when one laser beam is irradiated alone. This shows the advantage of relative low excitation powers of 2c2p excitation compared to those needed for three photon excitation.

Figure 3 demonstrates the possibility of imaging intrinsic protein fluorescence in living cells using 2c2pLSM. As expected, there is lower fluorescence intensity in the nucleus compared to the cytoplasm indicating different concentrations of tryptophan and, therefore, differences in protein concentration. Although a detailed conclusion about the tryptophan distribution inside the imaged cells cannot be drawn from these first pictures, they demonstrate the possibilities for further investigations. In particular the setup is ideally suited for performing fluorescence recovery after photo bleaching (FRAP) experiments which could yield information about the rate of protein syntheses and diffusion [20]. The difference in the fluorescence lifetime between nucleus and cytoplasm is caused by different tryptophan environments. The fluorescence lifetime of tryptophan is highly sensitive to its surrounding and many models have been proposed to explain this behavior [15]. It has been found that the conformation of tryptophan has a major effect on its lifetime. Especially different angles of rotation around the bonds between the mesomeric system of tryptophan and its acidic function have been proposed to have great effect on the lifetime. This kind of rotation determines the distance between the excitable π-system and the carbonyl as a possible quencher. Hence, different rotamers of tryptophan have different lifetimes. Of course, other polar molecules in direct vicinity also have great influence on the fluorescence lifetime of tryptophan as they can act as quenchers.

Apart from those rather sophisticated and detailed studies on the fluorescence lifetime of tryptophan containing proteins in solution a general qualitative statement can be made: [15] short lifetimes are found when the surrounding of tryptophan allows it to rotate freely, e.g. if it is not bound or bound in a short peptide, e.g. a tripeptide [21]. Long lifetimes are preferably found when tryptophan is bound in a constraining complex molecule like a big protein [22]. Therefore, it can be concluded that the fluorescence lifetime data from the nucleus reflects an excess of low molecular tryptophan whereas the longer lifetime in the cytoplasm reflects tryptophan in a high molecular surrounding. This result is in accordance with the known protein biosynthesis which takes place outside the nucleus.

Fluorescence lifetime measurements can provide detailed information about protein reactions. Figure 4 shows fluorescence decay data obtained after 2c2p excitation of the intrinsic fluorescence of avidin. The special property of avidin is, as widely known, its highly specific and strong binding to biotin. This binding mechanism has found broad application in numerous fields of biochemistry. It is used in immuno assays as well as in purification procedures. 2c2pLSM offers a minimal invasive multi-photon fluorescence tool to analyze protein reactions like the avidin biotin binding without labeling. This might be a useful tool for evaluating the success of a biochemical reaction or assay, to give just one example. Due to the minimal amount of sample needed it might even be used for high throughput screening using fast evaluation methods of the fluorescence lifetime [23, 24].

The origin of the reduction in fluorescence lifetime can be understood with respect to the crystal structure of the

Table 1 Parameters of a biexponential decay fitting the data shown in Fig. 3 best

biexponential fit	Avidin		avidin + biotin		ΔA
y_0	-0.005	+/- 0.004	-0.004	+/-0.002	
A_1	0.23	+/-0.03	0.69	+/-0.02	0.46
τ_1	0.55 ns	+/-0.02 ns	0.55 ns	+/-0.02 ns	
A_2	0.74	+/-0.02	0.26	+/-0.02	+0.48
τ_2	2.10 ns	+/-0.08 ns	2.10 ns	+/-0.08 ns	

A: preexponential factor, τ: decay constant

avidin biotin complex [25]. In total, avidin contains four tryptophans, two of which are located directly at the binding pocket of biotin. Hence, it seems very likely that the reduction in fluorescence lifetime of avidin upon binding of biotin is due to a quenching mechanism between biotin and these two tryptophans. This model is supported by the evaluation of the parameters obtained from the biexponential fit to the experimental data. The 1:3 ratio of the preexponential factor of the short and the long fluorescence lifetime can be interpreted as 1 tryptophan predominantly exhibiting a short lifetime and the other three predominantly showing a long fluorescence lifetime. The lifetimes are considered to be predominantly only, because it is unlikely that each of the tryptophans exhibits only a monoexponential fluorescence intensity decay [21, 22]. Nevertheless, addition of equimolar biotin leads to a significant shift in the preexponential factors of the two lifetime components. Each preexponential factor is shifted by a difference of about 0.5. Considering the normalization of the fluorescence decay data and the four tryptophans of avidin, this is consistent with a change in fluorescence lifetime from long to short of exactly two tryptophans per avidin molecule. Similar findings have been reported earlier although the measured fluorescence lifetimes differ from our results [26]. With respect to the crystal structure, these two are most likely the tryptophans located in the biotin binding pocket whose fluorescence is quenched when biotin binds. The remaining preexponential factor of 0.25 of the long lifetime component indicates that one of the remote tryptophans exhibits a long fluorescence lifetime, while the other one possesses a short fluorescence lifetime.

Conclusion

With 2c2pLSM we demonstrate a novel multi-photon excitation fluorescence microscope technique that uses photons at two different wavelengths. The advantages are an extended spectral range where e.g. the Ti:Sa laser can be used for excitation and imaging of UV fluorophores, a reduced photo damage compared to one photon techniques and the possibility of performing highly resolved fluorescence microscopy with standard objectives which are not transparent at the effective excitation wavelength in the UV range. Additionally, all advantages of multi-photon techniques like intrinsic 3D resolution and increased penetration depth into biological tissue also apply for 2c2pLSM. The excellent deep tissue imaging properties of 800 nm light compensate the less ideal properties of 400 nm light if higher light intensities at 800 nm and only low intensities at 400 nm are used.

2c2pLSM performed with 400 nm and 800 nm light is ideal for excitation of tryptophan at an effective excitation wavelength of 266 nm. With this technique the autofluorescence of MIN-6 cells was imaged. Different fluorescence lifetimes have been found for nucleus and cytoplasm. To demonstrate the applicability of the method to label free protein studies the binding of biotin to avidin was monitored. From the obtained fluorescence lifetimes it can be concluded that two tryptophan residues are located at the binding site of avidin are quenched by the binding of biotin.

The reported results prove that Two-Color Two-Photon Laser-Scanning Microscopy can be a useful tool for non labeling protein fluorescence studies as well as for *in vivo* cell imaging.

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