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Independence of Maximum Single Molecule Fluorescence Count Rate on the Temporal and Spectral Laser Pulse Width in Two-Photon FCS

Jörg Mütze · Zdeněk Petrášek · Petra Schwille

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Abstract We investigate the fluorescence emission characteristics of standard dye tetramethylrhodamine (TMR) in two-photon fluorescence correlation spectroscopy for different temporal and spectral properties of the femtosecond excitation pulses. After determining the second-order dispersion of our setup, including the microscope objective, a pulse stretcher was employed to control the temporal width at the location of the specimen. As expected, the fluorescence per molecule and therefore the signal-to-noise ratio of an FCS-measurement can be improved at constant average excitation power by altering either the temporal or spectral width of the excitation pulses. We found however, that the maximum achievable molecular brightness is largely independent of the temporal and spectral width in the regime analyzed. This observation confirms the current working hypothesis for two-photon fluorescence correlation spectroscopy that bleaching and saturation, and thus, the inherent properties of the dye system, are the dominant effects limiting the quality of measurements. As a practical consequence, elaborate optimization of temporal and spectral laser pulse width, e.g. by introducing pulse stretchers in the beam path, is less critical than previously expected.

Keywords Fluorescence · FCS · Two-photon excitation · Single molecule spectroscopy

J. Mütze · Z. Petrášek · P. Schwille (✉)
Biophysics group, Biotechnologisches Zentrum,
Technische Universität Dresden,
Tatzberg 47-51,
01307 Dresden, Germany
e-mail: pschwil@gwdg.de

Introduction

Fluorescence correlation spectroscopy (FCS) is a highly sensitive and versatile technique used for the study of dynamics and interactions of individual fluorescently labeled molecules in solution or living cells [1, 2]. Two-photon FCS, where the fluorophore is excited by simultaneous absorption of two photons, provides numerous advantages over conventional FCS, particularly for applications in complex environments such as cells and organisms. It exhibits an intrinsic restriction of the excitation along the optical axis, leading to relatively low scattering background and confinement of the inevitable photobleaching of dye molecules to the focal vicinity [3–5], and thus allows larger penetration depths in biological samples. It moreover offers the possibility to simultaneously excite several spectrally distinct dyes with a single laser wavelength for dual-color cross-correlation measurements [6, 7]. Employing two-photon cross-correlation analysis, it has recently been possible to reveal the complex binding stoichiometry of protein–protein binding reactions in a living cell, demonstration the power of FCS as a method for in situ proteomics [8, 9]. The size of the two-photon measurement volume is comparable to conventional FCS without the need for a confocal pinhole. In order to achieve the necessary photon fluxes inside the excitation volume in the sample, it is necessary to employ femtosecond laser pulses with a mean power in the order of a few mW. The main disadvantage of two-photon excitation (TPE) compared to one-photon FCS is the rather low signal-to-noise ratio, usually determined by the detected photon count rate per single molecule per unit time. This signal limitation has so far mainly been attributed to photobleaching and saturation effects [10–12], although detailed reports about the relevance of excitation pulse quality control have so far been lacking. Shorter pulses, which require a broadened

spectral profile of the excitation light, are usually considered superior [13, 14], because of their higher efficiency of true two-photon transitions to the first excited state, versus one-photon processes induced by infrared photons that could lead to transitions to higher excited states. On the other hand, saturation might put a limit to the positive effect of pulse shortening already at moderate intensities. It has been demonstrated [6] that the maximum fluorescence emission yield in FCS for common dye systems is crucially dependent on the excitation wavelength, an effect that points to the strong influence of competing transitions to higher excited states, which could in turn be responsible for enhanced photobleaching. This raises the question whether a similar careful optimization as for the wavelength might be required for the pulse width in order to improve the FCS signal. The objective of the present study is thus, to characterize the single molecule signal quality in two-photon FCS with respect to all of these excitation parameters, laser power and wavelength, but also temporal and spectral pulse width for the common fluorophore tetramethylrhodamine (TMR).

Materials and methods

The temporal analysis of fluorescence intensity fluctuations of molecules passing through an optically defined open observation volume is the key principle of FCS. By recording the auto-and cross-correlation functions $G(\tau)$ and $G_x(\tau)$ of the fluorescence intensity fluctuations, it is possible to reveal information on concentrations and kinetic characteristics of different species of labeled molecules. The autocorrelation function (ACF), which is defined as:

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}, \tag{1}$$

describes the self-similarity of fluorescence fluctuations $\delta F(t)$ after a lag time τ. It contains information on the particle number N in the effective detection volume ($N = 1/G(0)$) [15], and in the simplest case of freely diffusing molecules with negligible internal dynamics as observed here, its decay is parametrized by the diffusion time τ_D, i.e., the characteristic time the molecule spends in the excitation volume, which is dependent on the diffusion coefficient of the molecules and the size of the volume.

One of the key parameters of interest in FCS applications is the number of photons emitted per molecule per second, often termed molecular brightness η [16]. It has been shown that the signal-to-noise ratio and thus, the statistical quality of the FCS data, is directly related to the detected fluorescence count rate per molecule [17, 18]. The molecular brightness η can simply be calculated by dividing the overall fluorescence count rate $F(t)$ by the number of molecules N in the effective measurement volume, where N can be estimated from the ACF as $1/G(0)$.

The time-averaged fluorescence signal for two-photon excitation depends on the square of the excitation intensity $I_0(t)$ and the temporal width τ_p of the excitation pulses [3]:

$$\langle F(t) \rangle \sim \frac{1}{\tau_p} \langle I_0(t) \rangle^2. \tag{2}$$

It follows from this relation, that in the absence of saturation and other limiting effects, the fluorescence yield can be increased either by increasing the mean excitation intensity $I_0(t)$ or by shortening the duration of the excitation pulses.

Measurements were performed on a home-built two-photon FCS setup [3]. The beam of a mode-locked Titanium: Sapphire laser (Mira 900-F, Coherent, 76 MHz repetition rate) is expanded five times and relayed into an inverted microscope (IX70, Olympus). The laser power is varied using neutral density filters and measured in front of the microscope. After focussing the beam with a high-NA objective (60×, 1.2NA IR, water immersion, Olympus), the induced fluorescence is collected by the same objective. The fluorescence emission passes a dichroic beamsplitter and an emission filter and is detected by a fiber-coupled Avalanche Photodiode (PerkinElmer Optoelectronics) with a 100 μm aperture. The temporal width of the femtosecond pulses at the location of the specimen is measured with a commercial autocorrelator (Carpe, APE GmbH). The spectral width is varied by changing the position of an intracavity prism. Sample solutions of TMR (Molecular Probes) with concentrations in the nanomolar range were filled into sealable chambers, assuring a constant sample concentration during the measurements.

Results

The influence of the excitation power on the autocorrelation curve at a fixed wavelength and pulse width is shown in Fig. 1. The amplitude $G(0)$ of the correlation curve decreases at high excitation powers (Fig. 1a), indicating an increase in the particle number N and/or a change in the size of the effective detection volume. This effect has been explained previously as a result of saturation [10, 19]. An effect typical of photobleaching is shown in Fig. 1b, where the normalized correlation functions decay faster at higher powers. The dye molecules are being bleached while diffusing through the detection volume, thus leading to a decrease of the apparent diffusion time. The inset shows that the effective molecular brightness η initially increases nearly quadratically with excitation power, reaches its maximum value of η_{max}=34 kHz/molecule at 22 mW and slowly decreases at even higher powers.

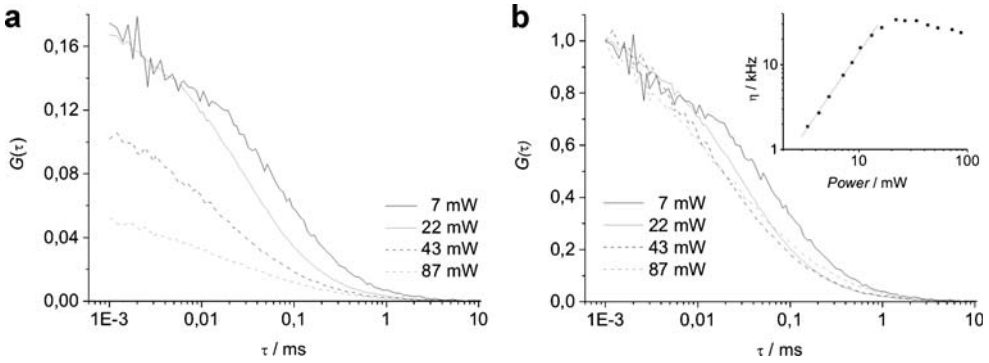

Fig. 1 The excitation power dependence of the autocorrelation function for a 14 nMol/l solution of TMR (λ=850 nm, τ_p=160 fs). With increasing laser power, both the amplitude $G(0)$ **a** and the diffusion time **b** decrease. In (**b**) the curves are normalized at τ=1 μs. A change in the shape of the normalized ACF for very high powers can be observed. The *inset*, plotted at a double logarithmic scale, shows the nearly quadratic dependence of the molecular brightness on the excitation power at low excitation powers up to 15 mW (slope= 1.87). At powers above 40 mW, η decreases, which has been attributed to fluorescence saturation and photobleaching [10, 11]

Figure 2 depicts the recorded two-photon spectral dependence of the maximum molecular brightness for different excitation powers but constant pulse width in the accessible range of the Titanium:Sapphire laser between 780 and 930 nm. Measurements were performed at a step size of 10 nm, corresponding to the spectral width of the excitation pulses. For each wavelength, η was recorded at various excitation powers while keeping the spectral and temporal widths of the pulses constant at 10 nm and 160 fs (FWHM), respectively. Uncorrelated background was corrected for as described previously [17, 20] for each individual measurement separately. The spectral variation of the maximum molecular brightness η_{max} dependent on excitation intensity is plotted in Fig. 3. This "maximum-η" excitation spectrum of TMR is qualitatively comparable to the published two-photon excitation spectrum of a similar dye Rhodamine B [13], indicating that the maximum achievable brightness is obtained by exciting with the wavelength corresponding to the maximum two-photon cross-section. For this reason, the excitation wavelength 850 nm was used for all subsequent measurements.

To control the temporal characteristics of the excitation laser pulses, it is necessary to characterize the pulse broadening by the optical elements in the beam path. Compared to the objective, the induced group velocity dispersion of all other optical elements, such as the beam expander, is negligible. The magnitude of the second order dispersion can be determined from the spectral and temporal pulse widths before entering and after passing the objective. A nearly transform-limited pulse of (120±4) fs and a spectral width of

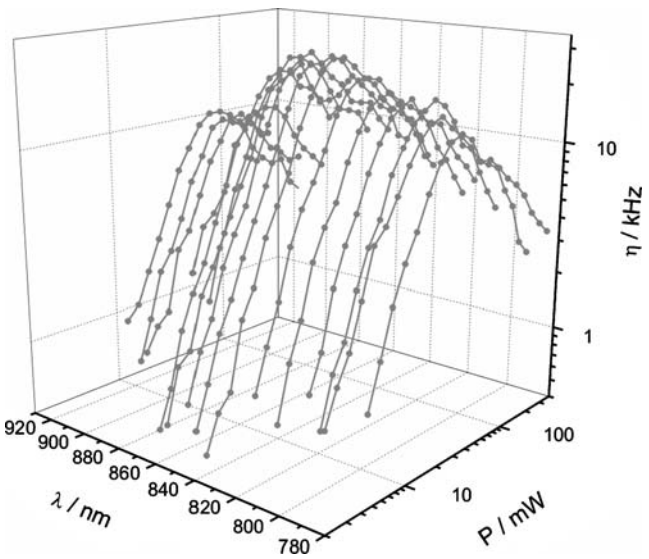

Fig. 2 Power and wavelength dependence of the molecular brightness in a two-photon FCS measurement for TMR. While the excitation pulse width (160 fs and 10 nm) for each wavelength is kept constant, the excitation power was varied

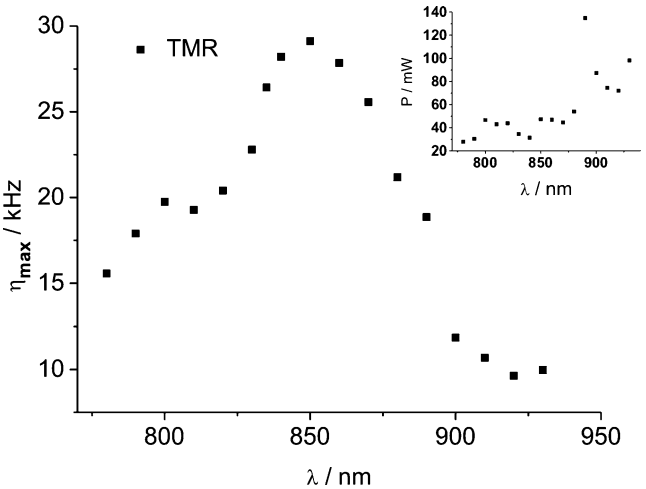

Fig. 3 Two-photon "maximum-η" excitation spectrum for TMR. The maximum count rate per molecule (η_{max}) at constant excitation pulse width (160 fs and 10 nm) for each wavelength is displayed. Compared to twice the wavelength of the excitation maximum for one-photon excitation (1,088 nm), the two-photon maximum is shifted to shorter wavelengths. The inset shows the excitation intensities at which the maximum η could be reached

$\Delta\lambda = (10.1 \pm 0.2)$ nm is broadened to (161±4) fs by the used objective, yielding a second order dispersion of (2,487± 650) fs^2. Comparable values have been obtained by others for similar objectives used in two-photon microscopy [21].

The unwanted pulse broadening in the microscopes objective due to group velocity dispersion was compensated using a method described by Fork et al. [22], which allows the creation of alterable negative group velocity dispersion using a pair of Brewster prisms (made of N-SF10 glass). The objectives dispersion was fully compensated at a total of 16 mm of the prism glass traversed and a prism to prism distance of 64 cm, which corresponds to a theoretical second order dispersion of −2,874 fs^2. Changing the amount of glass traversed in one of the prisms allowed independent adjustment of the pulse length (Fig. 4).

The inverse proportionality between the fluorescence intensity and the excitation pulse width (Eq. 2) was verified at pulse lengths between 128 and 227 fs (Fig. 5). At the excitation power of 7 mW, a fit to $a < F(t) > \sim 1/(\tau_p)^{\alpha}$ dependence for a total of 14 measurements resulted in an average exponent of $\alpha = 1.03 \pm 0.07$, in agreement with Eq. 2. At higher excitation powers, the exponent α decreased notably, indicating severe photobleaching or saturation in the focus of the laser beam.

The power dependencies of the molecular brightness η at constant spectral width ($\Delta\lambda$=11.4 nm) for different values of temporal pulse width (τ_p=128, 159 and 227 fs), and at constant temporal pulse width (τ_p=213 fs) for two different values of spectral width ($\Delta\lambda$=4.9 and 12.4 nm) are depicted in Figs. 6 and 7. At constant spectral width, an increase in brightness η for shorter pulses in the quadratic regime of the excitation powers could be observed in agreement with Eq. 2. An average increase in η of 48% (128 fs) and 29% (159 fs) compared to 227 fs was observed. At constant temporal width, the brightness η increased by 40% upon broadening the spectral width from 4.9 to 12.4 nm for excitation powers up to 10 mW (quadratic regime). In both cases, the highest achievable molecular brightness was found to be independent of either τ_p or $\Delta\lambda$, reaching a maximum value of 35 kHz/molecule. Similarly, the maximum brightness η

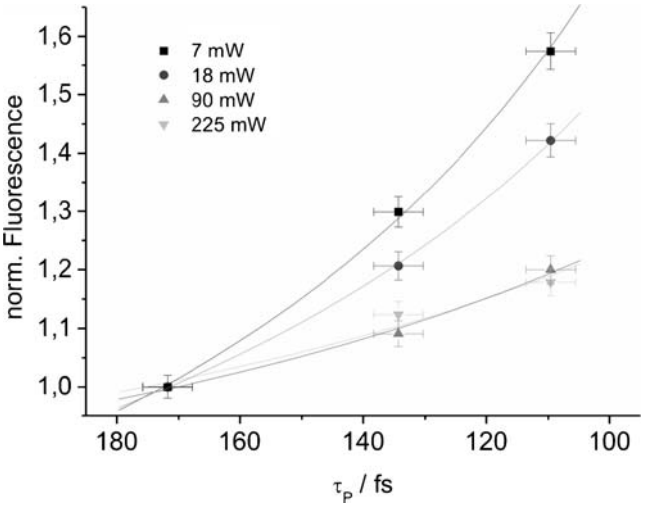

Fig. 5 Decreasing the pulse width for a 100 nMol/l TMR sample leads to an increase in the normalized fluorescence intensity. Data points were fitted to a $1/(\tau_p)^{\alpha}$ dependence resulting in an exponent of $\alpha = 1.03 \pm 0.07$ for an excitation power of 7 mW, in agreement with Eq. 2. For powers above 7 mW, the fluorescence increase is reduced, indicating the onset of photobleaching or saturation effects in the focus of the laser beam

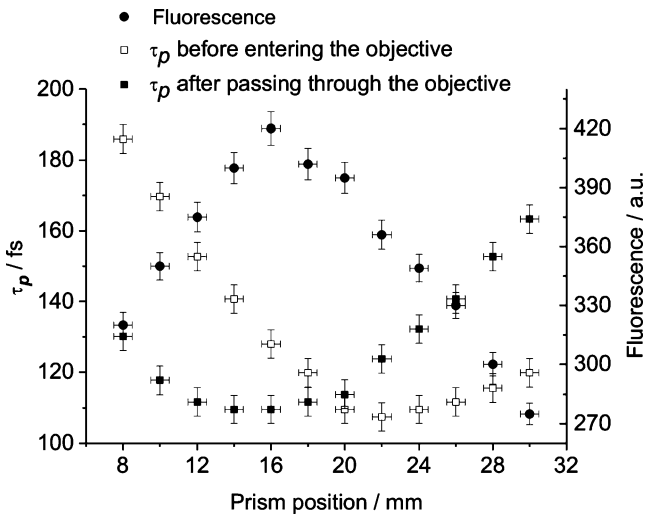

Fig. 4 Compensation of the group velocity dispersion using two prisms: by changing the amount of prism glass traversed, the amount of induced negative group velocity can be adjusted. In addition to the excitation pulse width before entering and after passing through the objective, the intensity of fluorescence generated in a solution of TMR is shown. The maximum fluorescence intensity coincides with the shortest pulses in the solution (Eq. 2)

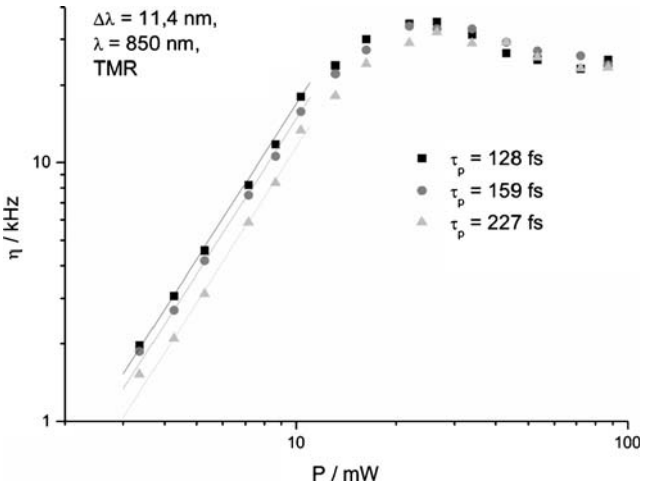

Fig. 6 Excitation power dependence of the molecular brightness η measured with two-photon FCS for TMR at three different excitation pulse widths. While keeping the spectral width constant at 11.4 nm, the pulse width was varied between 128 and 227 fs using the prism compressor described in the text. An increase in η with shorter τ_p (48% for 128 fs and 29% for 159 fs compared to 227 fs) can be observed in the quadratic regime of the excitation power dependence. For comparison, the quadratic slopes are plotted

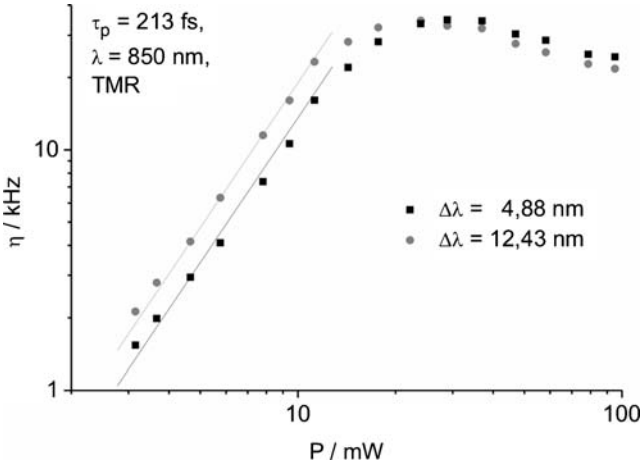

Fig. 7 Measured excitation power dependence of the molecular brightness η for TMR for two different spectral widths at a constant temporal pulse width of 213 fs. For excitation powers of up to 10 mW, an increase of 40% in η upon extending the spectral width from 4.9 to 12.4 nm could be observed. For comparison, the quadratic slopes are plotted

within the quadratic regime before onset of photobleaching or saturation is independent of τ_p or $\Delta\lambda$.

Conclusions

Two-photon pulsed excitation of fluorophores depends, apart from the excitation intensity and wavelength, on the temporal and spectral width of the femtosecond excitation pulses. In FCS, high fluorescence yields, quantified by molecular brightness η, are required for high signal-to-noise ratios. However, high excitation intensities generally needed to achieve high η can cause saturation and/or photobleaching, leading to distortions in autocorrelations curves. We have investigated the effect of the above mentioned parameters influencing two-photon excitation on molecular brightness, with the goal to find their optimal values (i. e. the values leading to the highest molecular brightness before the distortions become apparent) in the range accessible with a commercial infrared femtosecond laser and a relatively simple pre-chirping unit.

The measurements at different excitation wavelengths showed that there clearly is an optimal wavelength at which both the highest maximum molecular brightness η_{max}, and the highest molecular brightness within the quadratic range can be reached. This is the wavelength of maximum two-photon cross-section, since at constant excitation intensity within the quadratic range, this wavelength yields the highest maximum brightness.

This result also indicates that saturation of the S_0-S_1 transition is not the only factor limiting the maximum molecular brightness η_{max}. If this was the case, we would expect to reach the same saturation-limited value of maximum brightness at all excitation wavelengths. Of course, at wavelengths with low two-photon cross-section, higher excitation intensity would be needed to reach this value. We have, however, observed lower maximum brightness values at wavelengths with lower two-photon cross-section. Photobleaching via the population of other excited states is a likely cause, resulting in apparently shorter diffusion times at high excitation intensities (Fig. 1). With the yield of fluorescence and yield of photobleaching both being wavelength- and intensity-dependent, the excitation wavelength 850 nm gives the best ratio of fluorescence yield to photobleaching yield.

Having identified the optimal excitation wavelength, two additional parameters can be relatively easily modified with experimental setups commonly used for two-photon FCS: temporal and spectral pulse width of excitation. Both of them can, in principle, affect excitation and photobleaching efficiencies in the same way as the excitation wavelength, and it is therefore important to check whether an optimization procedure for these parameters is required, as for the wavelength, to obtain best results in FCS, i.e., if a combination of the two can be found where the molecular brightness is maximized.

At very low excitation intensities, the expected inverse proportionality between the fluorescence signal and the temporal width of the excitation pulses is observed. This results in an increase of molecular brightness for shorter temporal width in the quadratic regime, where experiments should be performed in order to minimize errors in the quantification of N and τ_D (Figs. 1, 6 and 7), and also coincides with observations in two-photon microscopy, where linear chirping of the excitation pulses was shown to lead to a reduction in fluorescence signal intensity [23]. However, the highest molecular brightness achievable is independent of the pulse length (Fig. 6) in the regime probed here.

Similar to the temporal width dependence, the molecular brightness exhibits a characteristic spectral width dependence. It is unclear whether this dependence is due to the broader range of accessible excitation wavelengths allowing multiple excitation pathways, or whether it is an effect of different pulse shape [24, 25]. As observed for the temporal pulse width, the spectral pulse width does not affect the maximum achievable brightness.

We conclude, that while there is an optimum excitation wavelength coinciding with the wavelength of maximum two-photon cross-section, the maximum achievable molecular brightness is independent of the temporal and spectral widths of the excitation pulses in the regime analyzed for TMR (similar results were obtained for eGFP, data not shown). This observation indicates that the temporal and spectral pulse widths affect the excitation and photobleaching yields in a similar manner, allowing for no settings with clear improvement of fluorescence to photobleaching yield ratio.

In practice, the total power used to illuminate the sample may be important for reasons other than prevention of saturation or photobleaching, for example, minimizing the phototoxicity and photodamage to biological specimens. Then, in order to minimize possible artifacts and damage to the sample due to intensity-dependent processes, it is desirable to keep the total laser irradiance as low as possible. For a given excitation power, the highest brightness is achieved by minimizing the temporal width and maximizing the spectral width of the excitation pulses. It can therefore be advantageous to implement the presented pre-chirping setup into a two-photon FCS system. In most cases, however, such a careful control and optimization of these pulse parameters is unnecessary, thereby simplifying the experimental setup and performance of two-photon FCS measurements and making them better adaptive for use in biological contexts.

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