Evaluation of a Femtosecond Fiber Laser for Two-Photon Fluorescence Correlation Spectroscopy

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ABSTRACT This work evaluates a femtosecond fiber laser for use in two-photon fluorescence fluctuation spectroscopy. Fiber lasers present an attractive alternative to Ti:Sapphire systems because of their compact size and portability. Autocorrelation of the second harmonic generation signal from the laser demonstrates that its stability is sufficient for two-photon fluorescence correlation spectroscopy. Fluorescence correlation spectroscopy autocorrelation traces were well fit by a Gaussian–Lorentzian squared model with a beam waist near the diffraction limit for the 810 nm wavelength. A photon counting histogram collected with this system also fit nicely to a single-species model, further demonstrating the quality of the focal shape. The authors conclude that the output from the femtosecond fiber laser is sufficiently stable and has a high enough quality beam shape for fluctuation fluorescence methods, and thus represents an effective, compact, readily portable two-photon excitation source. *Microsc. Res. Tech. 69:000–000, 2006.* © 2006 Wiley-Liss, Inc.

INTRODUCTION

Introduction and Instrumental Setup

The last decade has seen a renaissance of fluorescence fluctuation methods developed in the1970s (Hess et al., 2002; Magde et al., 1972). Among the reasons for its increasing popularity is its capability for resolving complex molecular interactions and microscopic dynamics in heterogeneous systems. The use of two-photon excitation has expanded the application of fluorescence correlation spectroscopy (FCS) and related methods through its intrinsic generation of the small observation volumes required to observe microscopic fluctuation phenomena (Bacia and Schwille, 2003; Berland et al., 1995; Denk et al.,1990; Schwille, 1999). This has led to increasing applications in the biological and material sciences.

The application of two-photon excitation in fluorescence fluctuation methods, however, requires a specialized femtosecond excitation source. While Ti:Sapphire systems are now much easier to use than in the past, they are still expensive and require a dedicated laboratory environment. Recently, powerful femtosecond fiber lasers have been developed that could be used in place of Ti:Sapphire systems for two-photon microscopy. They are relatively inexpensive, turnkey table-top systems requiring no more laboratory space or requirements than a table top air-cooled argon ion laser. Nevertheless, the utility of fiber lasers for two-photon FCS has not been fully explored. Fluctuations in the pulse width, repetition rate, or pulse energy could render these sources unsuitable for fluctuation measurements.

METHODS

Here we demonstrate FCS and photon counting histograms (PCH) (Chen et al., 1999) with a fiber laser (Femtolite F100, IMRA America, Ann Arbor, MI). This fiber laser has a pulse width < 150 fs and an average power of 100 mW. The average power at the sample was less than 30 mW for all studies reported here. The microscope and data collection system were similar to that described previously (Allen et al., 2004). The fluorescence signal was detected by an avalanche photodiode (Perkin Elmer SPCM AQR-14). In the present case, no excitation filter was used, and a 700 nm short pass dichroic (uvnirpc2, Chroma Tech., Rockingham, VT) was used to reflect the laser light to the sample and pass the fluorescence to the detector. Also, a custom 680 nm short pass filter was used to filter out the excitation light at the sample (e680sp-2p, Chroma).

RESULTS AND DISCUSSION

Noise Characteristics

To assess the stability of the fiber laser for FCS, it is necessary to rule out significant fluctuations in the square of the laser power on time scales longer than 10 μs. It was not possible to assess the stability of this laser on time scales shorter than this because of the after-pulsing characteristics of the avalanche photodiode available for the measurements. To check the stability of the laser source, we determined the autocorrelation of the second harmonic signal generated by focusing

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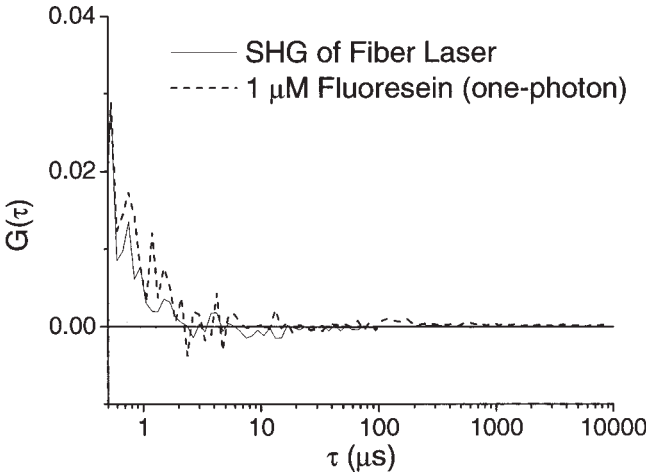

Fig. 1. Autocorrelation of SHG in a glass coverslip generated with the output from the fiber laser (solid line), and concentrated fluorescein fluorescence excited by a table-top argon-ion laser (dashed line). Autocorrelation decays in both cases are dominated by after-pulsing of the avalanche photodiode.

the fiber laser pulses in a glass cover slip (see Fig. 1). The autocorrelation was calculated as:

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

where $\delta(t) = I(t) - \langle I(t) \rangle$.

Figure 1 also shows for comparison the autocorrelation trace determined by excitation of a concentrated fluorescein solution with a table top argon ion laser (2201-20SL, JDS Uniphase, San Jose, CA). The autocorrelation trace generated by one-photon excitation with the argon ion laser shows similar short time scale fluctuation to the fiber laser. The autocorrelation does not decay to zero immediately for either excitation source, probably because of after-pulsing from our avalanche photodiode detectors. After-pulsing from avalanche photodiodes, such as the one used in this study, have been shown to contribute significantly to the autocorrelation function for time-scales as long as 10 μs (Becker, 2005).

FCS Characterization

Figure 2A shows two-photon-excited autocorrelation traces of two fluorescent dyes. We have fit these autocorrelations to the theoretical autocorrelation functions expected for a Gaussian–Lorentzian squared beam shape (Berland et al., 1995), where the axial dependence of the beam radius is described by

$$\omega^2(z) = \omega_0^2 \left(1 + \left(\frac{z}{z_R}\right)^2\right)$$

where ω_0 is the radial beam waist and $z_R = (\pi\omega_0^2/\lambda)$. The exact form of the autocorrelation function $G(\tau)$ has not been solved analytically for a Gaussian–Lorentzian focal volume, but Berland and coworkers have described a one dimensional integral that can be solved numerically to obtain $G(\tau)$ (Berland et al., 1995), and

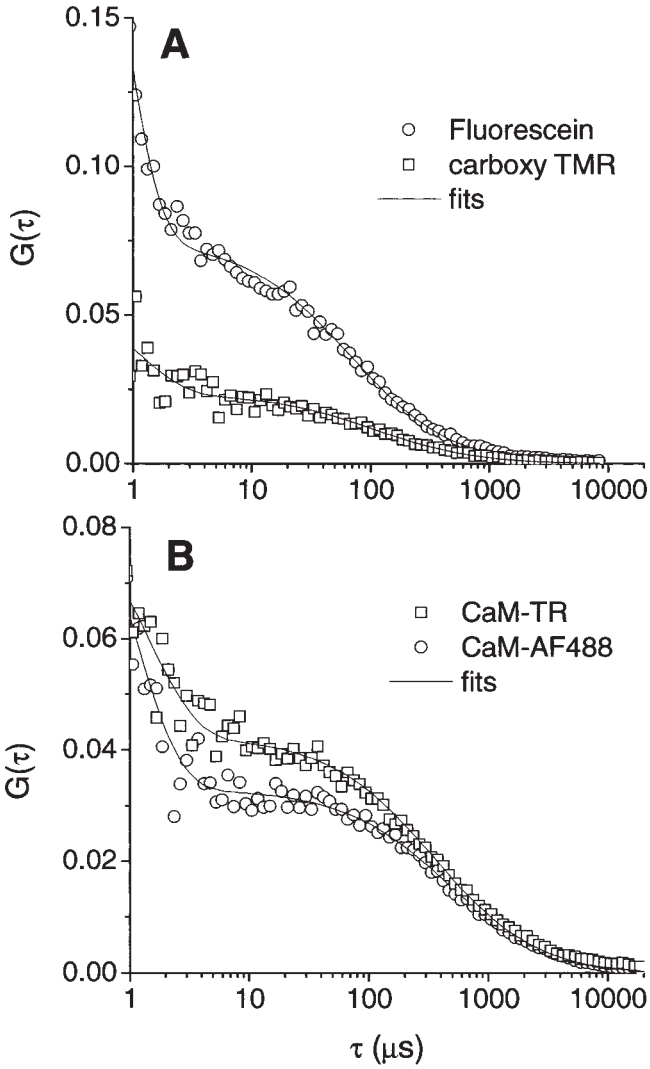

Fig. 2. Two-photon autocorrelation decays from (**A**) fluorescein and tetramethylrhodamine, and (**B**) calmodulin T34C labeled with two different fluorescent maleimides: Alexa Fluor 488 (CaM-AF488) and Texas Red (CaM-TR) (Molecular Probes, Eugene, OR). Fluorescein was measured in 0.1 M NaOH while the other solutions were measured in 10 mM HEPES, pH 7.4 with 100 μM calcium.

TABLE 1. FCS parameters

Sample	r_0 (μm)	D (μm²/s)
Fluorescein	0.38 ± 0.03	320[a]
c-TMR	Fixed	200 ± 100
CaM-AF488	Fixed	50 ± 10
CaM-TR	Fixed	60 ± 10

Errors were determined at one standard deviation using the support plane method (Johnson and Faunt, 1992).
[a](Coles and Compton, 1983; Daly et al., 1983).

that method was used here. Fast decay of the autocorrelation due to after-pulsing and triplet dynamics was accounted for by the addition of a short exponential to the fit as described by Widengren et al. (1995).

Given the diffusion coefficient of fluorescein (320 μm²/s (Coles and Compton, 1983; Daly et al., 1983), the fit to the autocorrelation for fluorescein yielded a value of

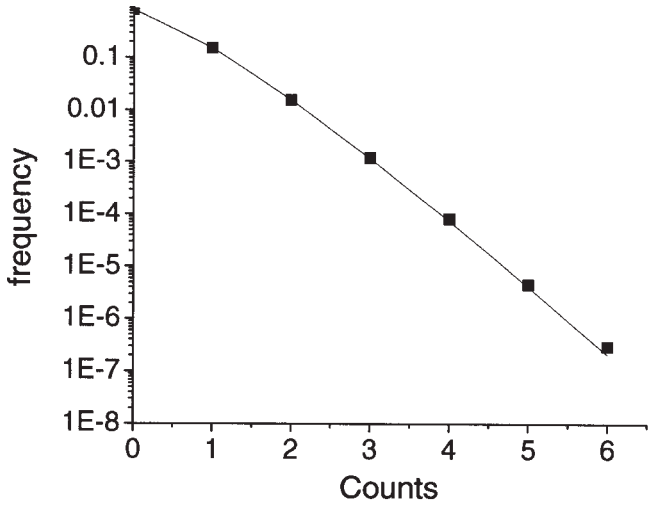

Fig. 3. Two-photon photon counting histogram for 5 nM fluorescein in 0.1 M NaOH. The line is the best fit to the Gaussian–Lorentzian squared model for a single species (Chen et al., 1999).

380 nm for the radial beam waist. This is in excellent agreement with the expected value for a diffraction limited focus. It was then possible to determine the diffusion coefficients of other species collected from the same optical setup. The measured diffusion coefficients are displayed in Table 1.

Figure 2B shows the two-photon autocorrelation traces of the T34C mutant of the calcium signaling protein calmodulin labeled with two different fluorescent dyes. Once again, the autocorrelation traces fit nicely to the Gaussian–Lorentzian model. The average diffusion coefficient for the two CaM species is 50 ± 10 $\mu m^2/s$, in good agreement with the value of 60 $\mu m^2/s$ (inferred from the diffusion time and Gaussian beam waist) measured by Schwille and coworkers (Kim et al., 2004).

PCH Characterization

In 1999, Gratton and coworkers introduced the novel PCH methodology with two-photon excitation for analyzing fluorescence fluctuations in terms of particle number and brightness (Chen et al., 1999) [A related method, known as fluorescence intensity distribution analysis, was introduced by Gall and coworkers (Kask et al., 1999)]. It has been observed recently that a poorly defined focal volume results in poor fits to the PCH (Huang et al., 2004). Thus, we have used the PCH method to evaluate the focal volume with fiber laser excitation. Figure 3 shows the PCH for fluorescein obtained with the fiber laser. The data fit nicely to the PCH for a single species, indicating that the focal volume obtained from the fiber laser is described well by the Gaussian–Lorentzian shape proposed for two-photon excitation (Chen et al., 1999).

CONCLUSIONS

We have demonstrated the utility of a femtosecond fiber laser for use in fluorescence fluctuation methods. Such lasers are compact, completely turn-key, table-top sources with significantly lower cost than Ti:Sapphire systems. The results reported here demonstrate that fiber laser systems are capable of stability as two-photon excitation sources at least comparable to a table-top argon ion laser used for one-photon fluctuation spectroscopy. Several examples demonstrated that fiber-laser two-photon FCS can be used to determine diffusion coefficients, and to determine a photon-counting histogram. The availability and ease of use of fiber lasers therefore holds excellent potential to enhance the convenience of two-photon FCS for bioanalytical and biomedical applications.

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