Resolution improvement in two-photon fluorescence microscopy with a single-mode fiber

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The dependence of spectral broadening of an ultrashort-pulsed laser beam on the fiber length and the illumination power is experimentally characterized in order to deliver the laser for two-photon fluorescence microscopy. It is found that not only the spectral width but also the spectral blue shift increases with the fiber length and illumination power, owing to the nonlinear response in the fiber. For an illumination power of 400 mW in a 3-m-long single-mode fiber, the spectral blue shift is as large as 15 nm. Such a spectral blue shift enhances the contribution from the short-wavelength components within the pulsed beam and leads to an improvement in resolution under two-photon excitation, whereas the efficiency of two-photon excitation is slightly reduced because of the temporal broadening of the pulsed beam. The experimental measurement of the axial response to a two-photon fluorescence polymer block confirms this feature.

OCIS codes: 170.2520, 060.2430, 060.4370, 110.2350, 190.4180.

1. Introduction

Two-photon fluorescence microscopy (TPFM) is a growing area in high-resolution imaging science.[1] Two-photon excitation arises because of the simultaneous absorption of two incident photons by a molecule, causing the transition of a ground-state electron to an excited state of the fluorophore. Because two photons are required for each transition, the probability of excitation is dependent on the square of the instantaneous incident radiation intensity, and thus an ultrashort-pulsed laser beam is usually needed for efficient excitation.[1] The quadratic dependence enables three-dimensional images to be obtained with submicrometer spatial resolution under two-photon excitation.[2,3] The introduction of optical fibers and fiber-optical components into conventional imaging systems in recent years[4–6] has provided additional advantages to existing modalities. Such advantages include the ability to place bulk optics and laser sources remotely from the sample[7,8] and to image specimens *in vivo*, since the excitation radiation can be delivered remotely.[9]

However, the introduction of a single-mode fiber to deliver an ultrashort-pulsed laser beam for two-photon excitation leads to physical complications. First, linear dispersion in the fiber core is induced as a result of group-velocity dispersion, which arises because different spectral components of a pulse travel at slightly different speeds.[10] Therefore any time delay in the arrival of spectral components leads to temporal broadening of the initial pulsed beam. Second, owing to the high peak power, an ultrashort-pulsed beam can result in nonlinear responses such as an intensity-dependent refractive index and intensity-dependent group-velocity dispersion.[10] These nonlinear responses lead to self-phase modulation and self-steepening, which cause spectral broadening and blue shifting of an ultrashort-pulsed beam.[10]

Both spectral and temporal broadening effects are of particular relevance in TPFM. First, under two-photon excitation, the fluorescence intensity signal I_f depends on the pulse width τ, the repetition rate F, and the average power P_{avg} of the laser beam by means of the following relation[11]:

$$I_f \propto \frac{(P_{\text{avg}})^2}{\tau F}. \tag{1}$$

The effect of the temporal broadening caused by linear dispersion (i.e., under the low-power condition) on

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Received 25 May 2001; revised manuscript received 19 November 2001.

0003-6935/02/101852-06$15.00/0

the efficiency of two-photon has been investigated by Wolleschensky *et al.* in detail.[12] Second, according to microscopic imaging theory under pulsed beam illumination,[13] the contribution of the shorter-wavelength components within a pulsed beam is stronger than that of the longer-wavelength components. Consequently, two-photon image resolution under ultrashort-pulsed beam illumination can be improved.[13,14] Such an improvement is small when the pulse width τ is larger than 10 fs.[14] However, because of the significant spectral broadening caused by self-phase modulation and blue shifting caused by the high-order nonlinear effect in a single-mode fiber, the improvement in two-photon image resolution under illumination of a pulse width of 100 fs may be observable. However, to our knowledge, this issue has not yet been addressed.

In this paper we present a detailed experimental investigation into the spectral broadening and shift of an ultrashort-pulsed laser beam propagating through a single-mode optical fiber and their effect on a TPFM system. The paper is divided into five sections beginning with the introduction in Section 1. Section 2 describes the experimental arrangement of the system. In Section 3 the experimental characterization of ultrashort-pulsed propagation in a single-mode fiber (spectral broadening and shifting) is presented. The measured results regarding the excitation efficiency and resolution improvement under two-photon excitation are given in Section 4. A conclusion is drawn in Section 5.

2. Experimental Arrangement

The experimental configuration of the two-photon fluorescence imaging system used in characterization is given in Fig. 1. An ultrashort-pulsed beam from a Ti:sapphire laser (Spectra Physics, Tsunami), operating at wavelength 800 nm, pulse width 100 fs, and repetition rate 80 MHz, was used as the illumination source. It was coupled, through a 0.25-N.A. microscope objective, O_1, into a length (1, 2, and 3 m) of a single-mode optical fiber with an operating wavelength at 785 nm. The fiber was placed in a chuck holder in an X, Y, Z positioner so that the fiber tip could be precisely positioned at the focus of the objective. Variation of the optical input power coupled into the fiber was achieved with a neutral-density filter, ND_1, placed in the beam path just before the coupling objective O_1. On exiting from the length of the fiber, the output laser beam was collimated by a second 0.25-N.A. microscope objective, O_2.

To record the spectral profile of the evolved pulse just after propagation through the fiber, a beam splitter, BS_1, was inserted into the path to direct a fraction of the laser beam into a spectrum analyzer connected to a digital signal analyzer. The transmitted beam from the beam splitter was coupled into a confocal scanning microscope (Olympus, FluoView). A variable neutral-density filter, ND_2, placed in the beam path just before the confocal scanning microscope was used to ensure that a constant input power to the instrument was maintained for a given input power and a given fiber length. Monitoring of the input and the output powers from the respective ends of the optical fiber was achieved with a portable optical powermeter.

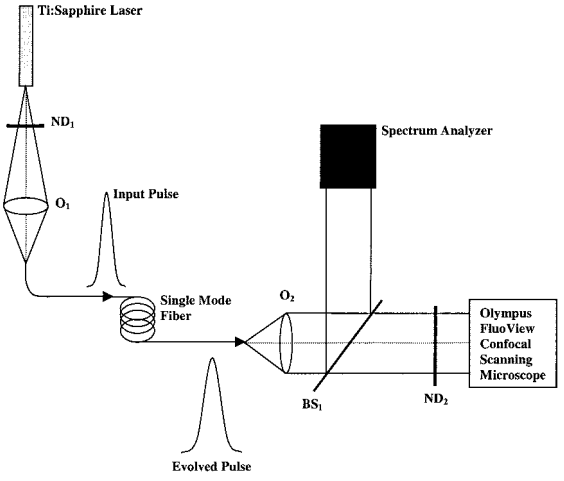

Fig. 1. Schematic diagram of the experimental setup. O_1 and O_2, 10× 0.25-N.A. microscope objectives; ND_1 and ND_2, neutral-density filters; BS_1, beam splitter.

The two-photon fluorescence efficiency under different conditions was measured from a uniform fluorescent polymer sample with the confocal scanning microscope. The polymer can be excited by two-photon absorption at wavelength 785 nm[15] with a coverslip-corrected objective of N.A. 0.85. The measurement was achieved by means of coupling the collimated beam from the fiber directly into the microscope through a side port of the scanning box. To ensure that the only signal measured was the fluorescence emission from our sample, a bandpass filter operating at wavelength 550 nm (±20 nm) was placed in front of the microscopes photomultiplier tube. The x–y images of the polymer surface were recorded for a range of input power. For each of these input power values, minor adjustments to the coupling stage were made to ensure that the coupling efficiency was maximized for any given input power. The average of the image intensity as a function of the input power gives the two-photon excitation efficiency.

To investigate the resolution performance in the two-photon imaging system with the fiber illumination and collection mechanism, the axial fluorescence response (or z direction) to the polymer was taken for the input power to the fiber in the range from 100 to 400 mW and a given fiber length. The detected fluorescence intensity signal was recorded at every 0.1-μm interval into the sample up to a depth of approximately 15 μm. All experimental values were averaged over five measurements.

3. Coupling Efficiency, Spectral Broadening, and Spectral Blue Shift

The laser coupling efficiency (the ratio of optical output power to optical input power) of a pulsed beam

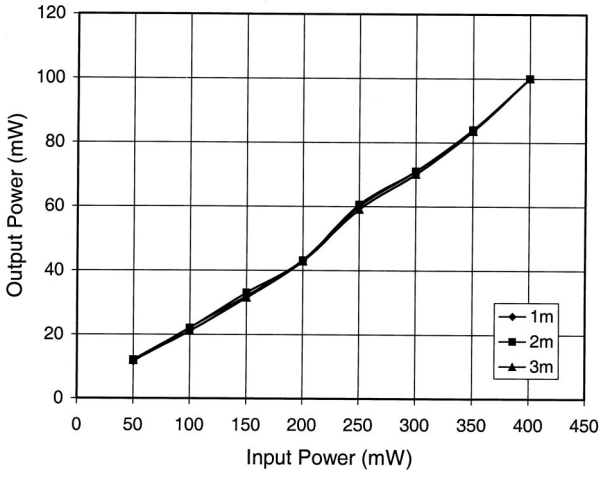

Fig. 2. Laser coupling efficiency for 1-, 2-, and 3-m lengths of the 785-nm single- mode fiber.

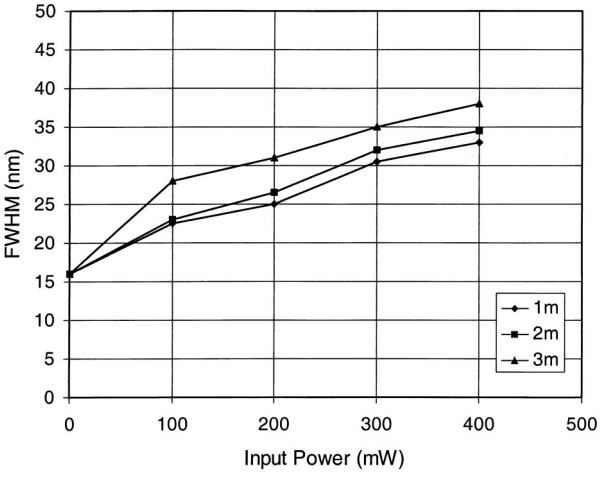

Fig. 4. Spectral FWHM as a function of the input power for 1-, 2-, and 3-m lengths of the 785-nm single-mode fiber.

into a single-mode fiber of lengths 1, 2, and 3 m, respectively, was measured for a range of the input power up to 400 mW and is plotted in Fig. 2. It can be seen that the coupling efficiency remains relatively the same for all three cases investigated, indicating that the coupling efficiency is independent of the length of the fiber used. It is interesting to note that a near-perfect linear relationship between the input fiber power and the output power tends to evolve into a nonlinear relationship when the input power exceeds 200 mW. The laser coupling efficiency in the linear region is approximately 24% for any given length of the fiber, whereas it is as high as 32% in the nonlinear region. This phenomenon may be caused by the spectral broadening and shift caused by the nonlinear linear response of the fiber, as shown in Figs. 3–5.

To characterize the spectral performance of an ultrashort-pulsed beam propagating through a single-mode fiber, we recorded spectral profiles of the illumination emerging from a length of the fiber, using a digital signal analyzer. For a given fiber length, spectral profiles were measured over a range of the input power up to 400 mW. The spectral FWHM for each recorded profile allows for an analysis of the spectral broadening effect. As an example, Fig. 3 shows the spectral profiles recorded by the digital signal analyzer for a fiber length of 3 m. For comparison, the initial pulse spectrum (the pulse spectrum produced by the laser) is included on the same axes, showing a spectral FWHM of 16 $\pm$ 0.2 nm. When the input power is 400 mW, the measured FWHM of the ultrashort-pulsed beam from the fiber is 38 $\pm$ 0.5 nm for a 3-m fiber, demonstrating a broadening factor of approximately 2.3.

The measured FHWM as a function of the input power is plotted in Fig. 4 for three fiber lengths. It is indicative from this plot that the broadening of the spectrum is approximately proportional to the fiber

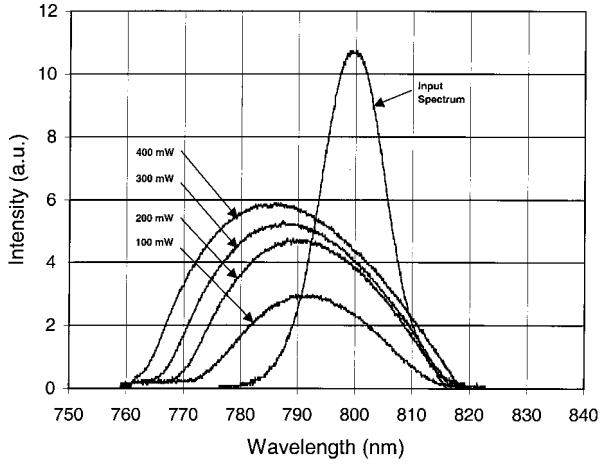

Fig. 3. Recorded spectral profiles of a pulse after propagation through a fiber length of 3 m for various input powers. The original pulse spectrum is included for comparison.

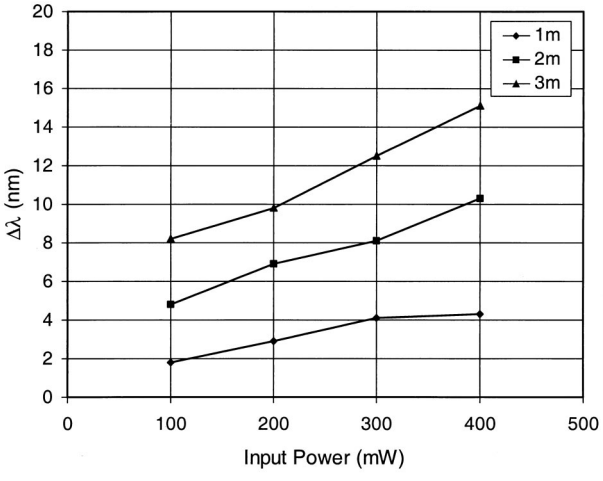

Fig. 5. Spectral blue shift, $\Delta\lambda$, as a function of input power for 1-, 2-, and 3-m lengths of the 785-nm single-mode fiber.

length for given input power. Furthermore, for a given fiber length, it is evident that the spectral broadening also increases with the input power. As an estimation, the nonlinear length L_N (Ref. 10) corresponding to the experimental condition is approximately 6 mm at the illumination power of 100 mW. It is conclusive from the experimental data that the spectral broadening is caused by self-phase modulation as discussed in Section 1.

Upon further observation of Fig. 3 it can be seen that, along with the spectral broadening, a shift in the central wavelength toward a short-wavelength region is also evident. The magnitude of this blue shift is depicted in Fig. 5. Here the blue shift $\Delta\lambda$ (defined as the shift in the central wavelength of the evolved pulsed beam with respect to the central wavelength of the input pulsed beam) is plotted as a function of the input power for the three fiber lengths investigated. For the case of a 3-m fiber with the input power of 400 mW, the spectral blue shift is 15 ± 0.5 nm. It is also clear from these measurements that the magnitude of the spectral shift is dependent not only on the length of the delivery fiber but also on the magnitude of the input power.

The spectral blue shift may result from a high-order nonlinear effect known as self-steepening, which is the intensity dependence of the group velocity when the temporal width of a pulse is as short as approximately 100 fs.[10] As a result, the peak of the pulsed beam travels at a slower speed than its wings, which results in a shift of the peak of the ultrashort-pulsed beam toward the trailing edge. This peak shift implies that the spectral broadening on the blue side is larger than that on the red side, since self-phase modulation generates blue components near the trailing edge.

Another consequence of the spectral blue shift is that the average wavelength of the spectrum is effectively reduced. Accordingly, it becomes close to that corresponding to the operating wavelength of the fiber at 785 nm, leading to a better coupling efficiency as observed in Fig. 2.

4. Resolution Improvement in Two-Photon Fluorescence Imaging

Although the relationship of the input power to the output power in Fig. 1 is approximately linear, there is a temporal broadening of the evolved pulsed beam.[10,12] As a result, the two-photon excitation efficiency may be reduced according to Eq. (1). The measured results of the two-photon excitation in a fluorescent polymer are presented in Fig. 6, which shows the log–log relationship of the detected fluorescence intensity as a function of the input power. The linear region of this chart is the verification that the detected fluorescence is indeed a result of two-photon excitation. In all three cases of the fiber length investigated, the gradient of the linear region was measured to be approximately 2.0 ± 0.1, indicating that a two-photon signal was measured, since the fluorescence intensity increases with the square of the excitation power. It is observed, however, that

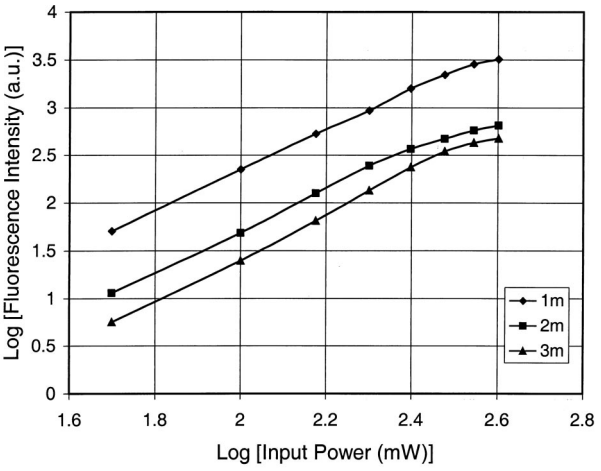

Fig. 6. Two-photon excitation efficiency for 1-, 2-, and 3-m lengths of the 785-nm single-mode fiber.

the two-photon efficiency in this linear relationship is degraded by a constant factor when the length of the fiber is increased. This result arises as a consequence of the linear dispersion effect acting on a pulse propagating in the fiber core. Under the experimental condition, the dispersion length L_D (Ref. 10) is approximately 17 cm. As a result, the temporal broadening of the pulse profile becomes pronounced as the distance of propagation increases. Because the two-photon fluorescence intensity signal is inversely proportional to the pulse width τ [see Eq. (1)], the two-photon efficiency drops off as the pulse width (i.e., the fiber length) increases.

Figure 6 also shows that for a given length of fiber there is a distinct bending away from the linear region as the input power increases. The gradient in this region tends to be lower than the gradient in the linear region of approximately 2.0. This phenomenon results from self-phase modulation and self-steepening in the presence of high input power. It is known that these effects lead to the temporal broadening of the ultrashort-pulsed beam,[10] which results in a further reduction of two-photon excitation efficiency in addition to the effect of the linear dispersion. The longer the fiber length, the more pronounced the effects of self-phase modulation and self-steepening.

As pointed out in Section 2, the axial response to the fluorescent planar polymer is a measure of axial resolution in two-photon fluorescence microscopy. The axial responses for the case of a 3-m fiber with the laser input power of up to 400 mW were measured and are shown in Fig. 7. It can be seen from this chart that the axial resolution, Δx, which is defined to be the distance between the 10% and the 90% points of the normalized fluorescence intensity, improves when we increase the input power coupled into the delivery fiber. This result is further demonstrated in Fig. 8 where the axial resolution is plotted as a function of the input power for fiber lengths of 1, 2, and 3 m. It clearly shows that a further slight im-

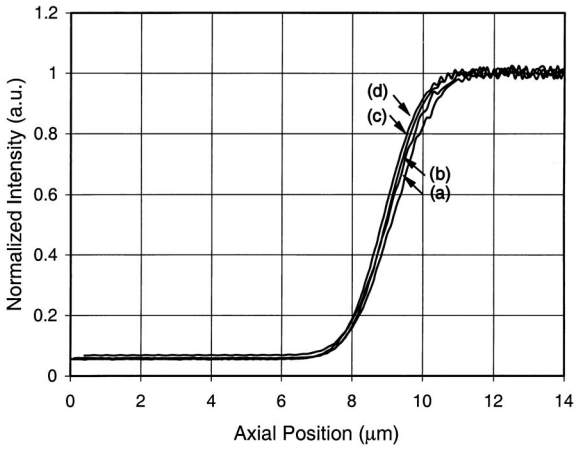

Fig. 7. Axial responses to the photobleaching polymer under two-photon excitation for a 3-m length of the 785-nm single-mode fiber with the input power of (a) 100, (b) 200, (c) 300, and (d) 400 mW.

provement in resolution is also achieved with increasing fiber length.

One of the reasons for the improvement in axial resolution in Figs. 7 and 8 is the spectral broadening and blue shift observed in Figs. 4 and 5. According to microscopic imaging theory,[13,14] for pulsed beam illumination, the contribution from short-wavelength components is stronger than that from long-wavelength components because the diffraction effect is inversely proportional to the illumination wavelength.[16] Consequently, imaging resolution can be improved for pulsed beam illumination. The broader the spectrum of a pulsed beam, and the further the peak is blue shifted, the larger the resolution improvement. For a spectral width of 40 nm as observed in Fig. 4, the resolution improvement can be as great as 7–8%.[14] If an ultrashort-pulsed beam exhibits not only the spectral broadening but also the spectral blue shift, the resolution improvement caused by the short-wavelength components should be as great as 9–10%, close to those listed in Figs. 7 and 8.

It should be pointed out that the spectral broadening is caused by self-phase modulation. Such a phase change may also affect the resolution behavior along the focal depth under two-photon excitation and should be theoretically explored, which is, however, not within the scope of this paper.

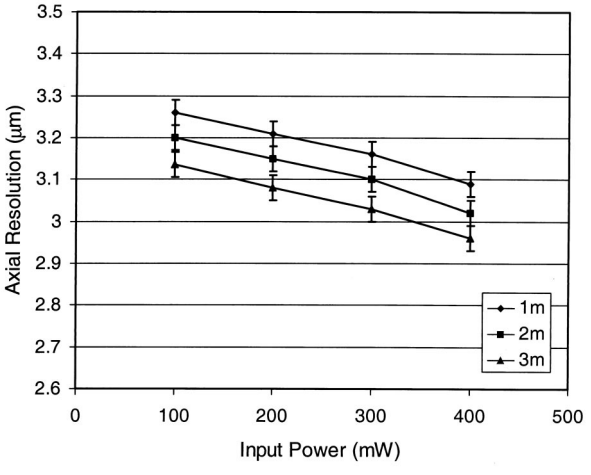

Fig. 8. Axial resolution Δx as a function of the input power for 1-, 2-, and 3-m lengths of the 785-nm single-mode fiber.

5. Conclusion

It is necessary to understand the significance of the results presented in this paper for practical two-photon fluorescence microscopy with a single-mode fiber for illumination and collection. For most biologic samples, an illumination power of 5–10 mW is needed after an imaging objective. For a laser beam at a wavelength of 800 nm, the overall transmission efficiency of scanning optics and an objective in a scanning optical microscope is usually less than 15%. Therefore an ultrashort-pulsed laser beam of power 200–300 mW should be coupled into a fiber, provided that the laser coupling efficiency is 25%. In this range we have demonstrated that the spectral property of an ultrashort-pulsed beam propagating through a single-mode fiber can be altered by the effect of linear dispersion and nonlinear responses such as intensity-dependent refractive index and intensity-dependent group velocity in the fiber. It has been found that the spectral broadening and spectral blue shift increase with the fiber length and the input power. These spectral changes are advantageous in TPFM consisting of a single-mode fiber for delivering an ultrashort-pulsed beam because image resolution is improved although the two-photon excitation is slightly reduced.

The authors thank the Australian Research Council for its support and acknowledge the useful discussions with X. Gan.

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