Compact two-photon fluorescence microscope based on a single-mode fiber coupler

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We present a two-photon fluorescence microscope based on a three-port single-mode optical fiber coupler. It is found that the coupler behaves as a low-pass filter that can deliver an ultrashort-pulsed laser beam of as much as 150 mW of power in the wavelength range from 770 to 870 nm as well as collect a two-photon fluorescence signal in the visible range. As a result of using the fiber coupler, the new two-photon imaging system exhibts a number of advantages, including a compact arrangement, freedom from vibration from lasers and electronic devices, self-alignment, reduction of multiple scattering, and an enhanced optical sectioning effect. The effectiveness of the new instrument is demonstrated with a set of three-dimensional images of biological samples. This instrument may make two-photon fluorescence endoscopy possible for *in vivo* medical applications. © 2002 Optical Society of America

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Since its inception,[1] two-photon fluorescence microscopy has rapidly emerged as an important technique for three-dimensional imaging of biological specimens. This emergence can be attributed to the advantages that two-photon excitation offers over single-photon excitation, which include an inherent optical sectioning property, confinement of photodamage to the focal region, and greater penetration depth into a sample.[1] One of the greatest restrictions of the technique in *in vivo* applications, however, is the use of complicated pulsed lasers and bulk optics. The introduction of optical fibers and fiber components into an imaging system[2–5] overcomes this physical limitation and provides the ability to image specimens *in vivo*, since the excitation radiation can be delivered to a remote sample. Recently, we reported on the operation of two-photon fluorescence microscopy consisting of a single-mode fiber for the delivery of an ultrashort-pulsed beam.[6] It has been found that, because of the nonlinear effects of self-phase modulation and self-steepening in the fiber, image resolution in this system can be improved.[6]

To achieve a cost-effective compact arrangement for two-photon fluorescence microscopy, one can use a multiport fiber coupler to replace bulk optics for illumination delivery and signal collection. Although multiport fiber couplers have been used in scanning differential interference contrast microscopy,[7] confocal reflection microscopy,[2,8] confocal interference miscroscopy,[9] and double-pass confocal reflection micrscopy,[10] to our knowledge there has been no report on using a fiber coupler for two-photon fluorescence microscopy. The difficulty with using a fiber coupler in this manner arises from the fact that the sepration of the excitation and fluorescence wavelengths is so large that a fiber coupler may not transmit the two wavelengths efficiently. In this Letter we present a compact two-photon fluorescence microscope that implements a three-port single-mode fiber coupler designed for an infrared wavelength. In addition to compactness, our new system is self-aligning because illumination delivery and signal collection use the same port. Since the small fiber aperture acts as a confocal pinhole, image resolution in this new system is higher than that in conventional two-photon fluorescence microscopy without a pinhole.[1]

The experimental arrangment of the new two-photon fluorescence microscope is given in Fig. 1. Ultrashort optical pulses generated from a turnkey Ti:sapphire laser (Spectra Physics Mai Tai) of wavelength range 730 to 870 nm, pulse width 80 fs, and repetition rate 80 MHz were used as the illumination. This laser beam was coupled into a port (port 3) of a three-port single-mode fiber coupler via a 0.25-N.A. objective, O_1. Rotation of a neutral-density filter, ND, placed before objective O_1 allowed variation of the input power. The output beam from port 1 of the coupler was collimated by a second 0.25-N.A. objective, O_2, and then passed through a variable aperture, AP, to fill the back aperture of the imaging objective, O_3 (0.85 N.A. 40×, ∞/170). The emitted fluorescence from a sample was collected by objective O_3 and returned via the same optical path used for pulse delivery. The signal was delivered via port 2 of the coupler into a photomultiplier tube. We placed a
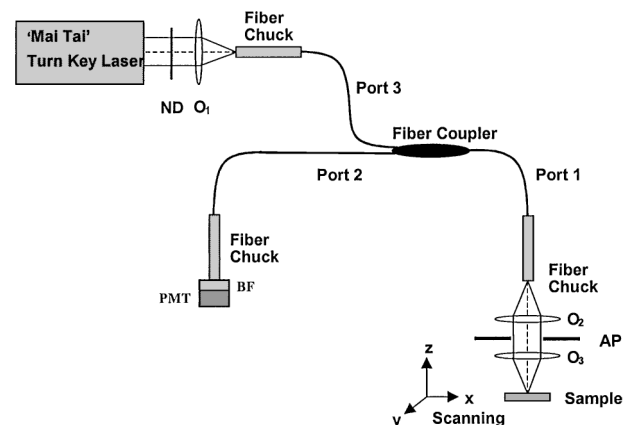

Fig. 1. Schematic diagram of the two-photon fluorescence microscope with a fiber coupler. PMT, photomultiplier tube. Other abbreviations defined in text.

bandpass filter, BF, operating at wavelength 550 nm (± 20 nm) in the beam path to ensure that only the fluorescence signal was detected. Each port of the fiber coupler was placed in a chuck holder in an x−y−z micropositioner to allow precise positioning of the fiber tip at the focus of an objective.

To obtain optimum delivery of the pulsed laser beam, we used a fiber coupler (Newport) designed for operation at wavelength 785 nm with an equal splitting ratio. The length of each arm of the coupler is 1 m. The measured coupling efficiency from ports 2 and 3 to port 1 was 20−38% in the wavelength range 770−870 nm. This property implies that laser power of as much as 80−150 mW in this wavelength range can be delivered to a microscope objective. As most commercial objectives have a transmittance of approximately 10% in the infrared region and the ultrashort-pulsed beam is broadened to approximately a few picoseconds after it transmits through the coupler, this coupler is capable of delivering a laser beam of sufficient power for two-photon excitation.[11] However, in the visible range in which two-photon fluorescence falls, the coupling efficiency of this coupler may be reduced, and an equal splitting ratio between ports 2 and 3 may not necessarily be maintained. To confirm these features, we measured the mode profile and the coupling efficiency at ports 2 and 3 while port 1 was illuminated by a cw beam of wavelength 532 nm (Spectra Physics Millennia). It was found that the field distribution from ports 2 and 3 is a single-mode profile, which is consistent with the estimation based on the core size and the N.A. of the coupler in the visible range. The coupling efficiency at port 2 was approximately 1%, with a splitting ratio of 90:10 between ports 2 and 3. As a result, using port 2 for the signal collection of two-photon fluorescence and port 3 for delivery of the pulsed beam means that the coupler acts as a low-pass filter and that the strength of the two-photon fluorescence signal can be maximized.

The depth discrimination of the new imaging system was characterized by measurement of the axial response to a thin fluorescent layer. The layer was produced by evaporation of a mixture of AF-50 dye and isopropanol alcohol on a coverslide. The sample was excited by two-photon absorption at a wavelength of 800 nm. The measured fluorescence intensity as a funciton of the power input to port 3 of the coupler is shown in Fig. 2 on a log−log scale. It can be seen that the gradient of the curve is approximately 2.0 ± 0.1, indicating that the fluorescence signal varies with the square of the input power, as expected for two-photon excitation.

Typical axial responses are shown in inset (a) of Fig. 2. It is shown that the FWHM of the axial response measured in the new two-ohton fluorescence microscope, Δz, is approximately 2.1 μm for an input power of 200 mW, which is reduced by approximately 30% compared with that measured by a large-area detector without a pinhole.[6] The decrease in the FWHM indicates the enhancement of the optical sectioning effect and therefore the improvement in axial resolution. In this case, the resolution improvement results from the aperture of port 1, which acts as an effective

confocal aperture.[12] The magnitude of the resolution improvement is actually less than the theoretical prediction[12] of 43% based on the assumption that the ratio of fluorescence to excitation wavelengths is 0.5. However, in our experiments an illumination wavelength of 785 nm was used, and the wavelength of the emitted fluorescence is approximately 540 ± 10 nm. As a result, our measurement of the axial-resolution improvement is in good agreement with the theoretical prediction based on a fluorescence-to-excitation wavelength ratio of approximately 0.7. In inset (b) of Fig. 2, the dependence of FWHM Δz on the incident power to port 3 is depicted, showing that the resolution is improved as the input power is increased. This feature is caused by the fact that the spectrum of the pulsed beam is broadened and blueshifted because of self-phase modulation[13] and self-steepening,[13] respectively, in a fiber.[6]

To demonstrate the three-dimensional imaging capability of the new system, in Fig. 3 we show a series of image sections taken at a 1-μm-depth steps into an ensemble of 10-μm fluorescent polymer microspheres. Figure 4 displays a series of image sections of the autofluorescence signal from sea algae, *Griffithsia*, demonstrating the applicability of the instrument for biological study. Both image sets exhibit high contrast and the pronounced optical sectioning property of the system.

Our result leads to the possibility of two-photon fluorescence endoscopy if an ultrashort-pulsed fiber laser and gradient index rod lenses are used. A further advantage of the effective pinhole aperture of the coupler is that the signal strength resulting from multiple scattering may be significantly reduced if a tissue medium is imaged under two-photon
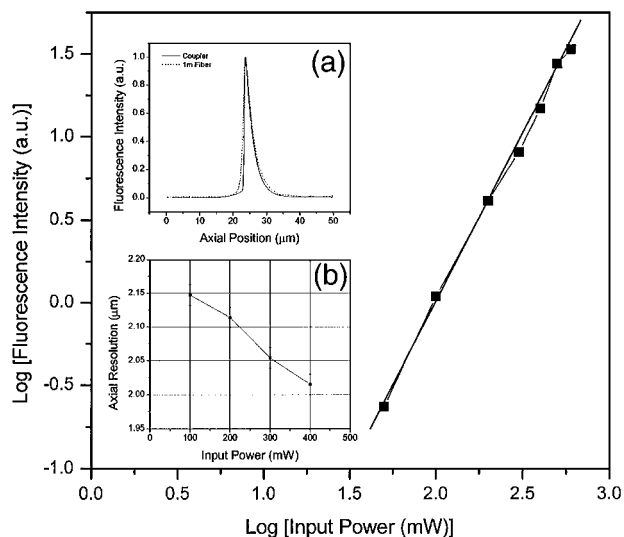

Fig. 2. Two-photon excitation efficiency of the two-photon fluorescence microscope with a fiber coupler. Inset (a) shows the axial responses to a thin fluorescent layer in the system when a coupler is used for (solid curve) an input power of 200 mW and (dotted curve) 1-m fiber delivery system employing a large-area detector without a pinhole. Inset (b) gives the dependence of the width of the axial response on the excitation power.
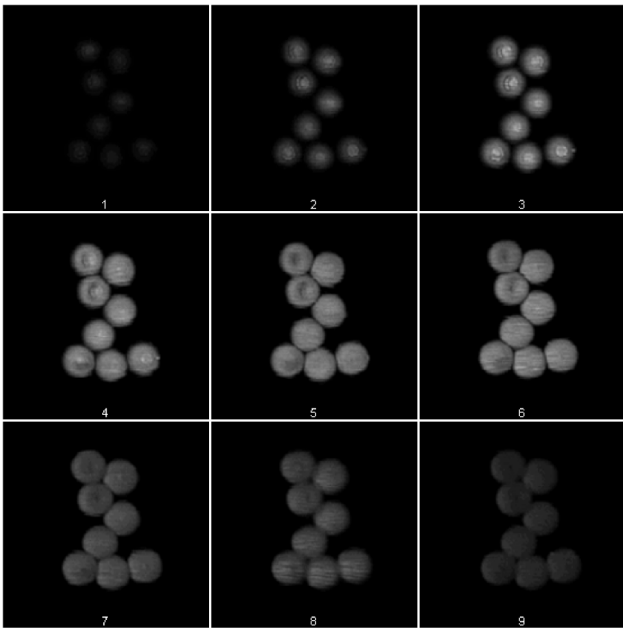
Fig. 3.   Images of 10-μm-diameter fluorescent polymer microspheres in a two-photon fluorescence microscope with a fiber coupler.   The size of the slices is 60 μm $\times$ 60 μm, and the slice spacing is 1 μm.   The excitation power is 8 mW at the focus.
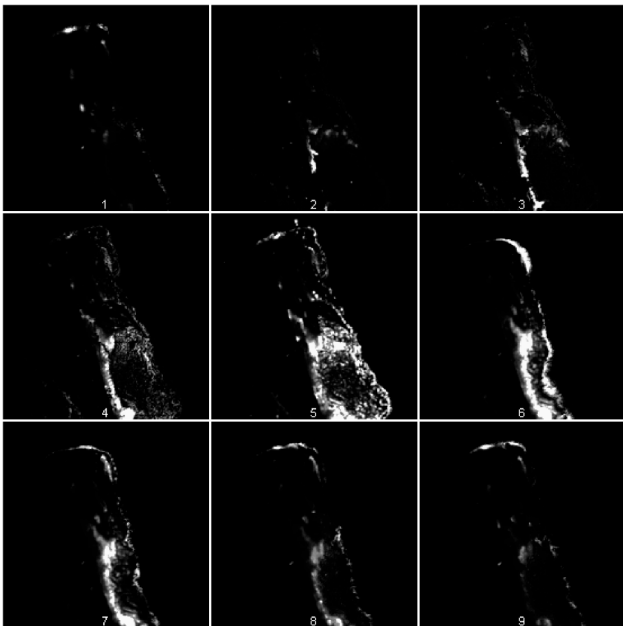

Fig. 4.   Images of *Griffithsia* sea algae in a two-photon fluorescence microscope with a fiber coupler.   The size of slices is 150 μm $\times$ 150 μm, and the slice spacing is 1 μm. The excitation power is 8 mW at the focus.

excitation.[14] This feature, together with others demonstrated in this Letter, makes it possible for two-photon fluorescence endoscopy to serve as a new tool for *in vivo* medical applications, including surgical biopsy and early cancer detection.   Such an endoscopic device will be an important complementary instrument of optical coherence tomography,[15,16] because the device provides a different contrast mechanism that shows sample functionality.   Further, our result implies that it is possible to achieve simultaneous operation of two-photon fluorescence microscopy and optical coherence tomography by use of a fiber coupler.

In conclusion, we have demonstrated the feasibility of compact low-cost two-photon fluorescence microscopy that uses a single-mode fiber coupler. The coupler acts as a low-pass filter for an infrared ultrashort-pulsed laser beam, so that both the delivery of the pulsed beam and the collection of two-photon fluorescence can be optimized.   The aperture of the fiber is an effective confocal pinhole, which leads to self-alignment, the reduction of multiple scattering, and an enhanced optical sectioning effect for high-resolution three-dimensional two-photon fluorescence imaging.   However, it should be pointed out that, in practice, backreflection from the confocal fiber aperture can be reduced through antireflection coating or angle cleaving to improve the signal-to-noise ratio of the system.

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