Two-photon microscopy with wavelength switchable fiber laser excitation

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Abstract: Two-photon scanning fluorescence microscopy has become a powerful tool for imaging living cells and tissues. Most applications of two-photon microscopy employ a Ti:sapphire laser excitation source, which is not readily portable or rapidly tunable. This work explores the use of two-photon fiber laser excitation (TP-FLEX) as an excitation source for scanning two-photon microscopy. We have further demonstrated the use of a photonic crystal fiber (PCF) for facile tuning of the excitation wavelength over the range from 810 nm to 1100 nm. We generated two-photon fluorescence images at excitation wavelengths from 850 nm to 1100 nm detected on a scanning-stage microscope. By PCF wavelength tuning the dye BODIPY fl was selectively excited at 1000 nm whereas MitoTracker red was excited preferentially at 1100 nm. We discuss the potential for fiber laser sources coupled with PCF wavelength tuning as an attractive tunable excitation source for two-photon scanning fluorescence microscopy.

OCIS codes: (180.0180) Microscopy; (170.0170) Medical optics and biotechnology; (060.4370) Nonlinear optics, fibers.

References and links

1. W. Denk, J. H. Strickler and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," Science **248**, 73-76 (1990)
2. W. R. Zipfel, R. M. Williams and W. W. Webb, "Nonlinear magic: multiphoton microscopy in the biosciences," Nat Biotechnol **21**, 1369-1377 (2003).
3. A. Diaspro, Ed., *Confocal and Two-Photon Microscopy. Foundations, Applications, and Advances*, (Wiley-Liss, New York, 2002).
4. K. König, "Multiphoton microscopy in life sciences," J Microsc. **200**, 83-104 (2000).
5. C.-Y. Dong, K. H. Kim, C. Buehler, L. Hsu, H. Kim, P. T. C. So, B. R. Masters, E. Gratton and I. E. Kochevar, "Probing deep-tissue structures by two-photon fluorescence microscopy," Emerging Tools for Single-Cell Analysis 221-237 (2000).
6. J. C. Knight, T. A. Birks, P. S. J. Russell and D. M. Atkin, "All-Silica Single-Mode Optical Fiber with Photonic Crystal Cladding," Opt. Lett. **21**, 1547-1549 (1996)
7. N. Nishizawa and T. Goto, "Widely wavelength-tunable ultrashort pulse generation using polarization maintaining optical fibers," IEEE J. Sel. Top. Quantum Electron. **7**, 518-524 (2001).
8. M. A. Albota, C. Xu and W. W. Webb, "Two-photon fluorescence excitation cross sections of biomolecular probes from 690 to 960 nm," Appl. Opt. **37**, 7352-7356 (1998).
9. G. A. Blab, P. H. M. Lommerse, L. Cognet, G. S. Harms and T. Schmidt, "Two-photon excitation action cross-sections of the autofluorescent proteins," Chem. Phys. Lett. **350**, 71-77 (2001)
10. C. Xu, W. Zipfel, J. B. Shear, R. M. Williams and W. W. Webb, "Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy," Proceedings of the National Academy of Sciences of the United States of America **93**, 10763-10768 (1996).
11. C. Xu and W. W. Webb, "Measurement of Two-Photon Excitation Cross Sections of Molecular Fluorophores with Data from 690 to 1050 nm," J. Opt. Soc. Am. B **13**, 481-491 (1996).
12. G. P. Agrawal, *Nonlinear Fiber Optics*, (Academic, San Diego, CA,2001).
13. J. C. Knight, J. Arriaga, T. A. Birks, A. Ortigosa-Blanch, W. J. Wadsworth and P. S. Russell, "Anomalous dispersion in photonic crystal fiber," IEEE Photon. Technol. Lett. **12**, 807-809 (2000).

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14. A. Diaspro, G. Chirico, F. Federici, F. Cannone, S. Beretta and M. Robello, "Two-photon microscopy and spectroscopy based on a compact confocal scanning head," J. Biomed. Opt. **6,** 300-310 (2001).
15. M. W. Allen, R. J. B. Urbauer, A. Zaidi, T. D. Williams, J. L. Urbauer and C. K. Johnson, "Fluorescence Labeling, Purification and Immobilization of a Double Cysteine Mutant Calmodulin Fusion Protein for Single-Molecule Experiments," Anal. Biochem. **325,** 273-284 (2004).

1. Introduction

Since it was first introduced in 1990 [1], there has been rapid growth in applications of two-photon microscopy [2-5]. The capability to excite visible or UV transitions of common biological fluorophores with near-infrared excitation significantly enhances a variety of applications in the biological sciences, including live-cell and tissue imaging. The highly localized character of two-photon excitation and the use of near-infrared wavelength help to minimize cellular damage and significantly reduce autofluorescent background present in confocal microscopy.

So far, a major barrier to expanded application of two-photon laser scanning microscopy (TPLSM) has been the requirement for a bulky and expensive ultrafast Ti:Sapphire laser system. Recent developments in fiber-optic devices for communication systems have made ultrafast near-infrared fiber lasers available. Not only are fiber-optic lasers less expensive than Ti:Sapphire laser sources, they are easily portable, highly reliable and robust. The recent progress in nonlinear photonic crystal fibers (PCF's) [6], introduces the possibility of wavelength tunability based on the effect of soliton self-frequency shift (SSFS) [7]. In this paper, we demonstrate the feasibility of two-photon imaging with selectable excitation wavelength based on wavelength switching in a PCF within the range from 810 nm to 1100 nm. In this wavelength tuning process, the alignment of the excitation beam into the microscope is not altered during wavelength tuning. We have termed this methodology two-photon, fiber laser excitation (TP-FLEX).

Wavelength switching in a PCF makes possible selective excitation of two-photon fluorophores. To date, there have been relatively few reported applications exploiting selective excitation of two-photon fluorophores [8-11]. Indeed, overlap of the two-photon excitation profiles for different fluorophores has been viewed positively because it allows simultaneous imaging of multiple fluorophores for some combinations of fluorescent dyes. Nevertheless, there are many applications in which it would be beneficial to selectively excite fluorophores. Image recording at different excitation wavelengths will permit applications such as TPE of ratiometric fluorescent ion indicators and determination of intracellular Ca^{2+} levels, for example. Similarly, Förster resonance energy transfer (FRET) requires the selective excitation of donor and acceptor fluorophores for unambiguous interpretation, a requirement that has limited the application of FRET methods in two-photon microscopy. TP-FLEX allows for optimal and selective excitation of multiple fluoropohres with a single fiber coupled laser source. In addition, PCFs are capable of generating wavelength-shifted solitonic radiation with longer infrared wavelengths (>1050 nm) that are not easily obtained with Ti:Sapphire lasers. This extended capability promises to extend the application of two-photon excitation to red and near-infrared emitting fluorophores. The increased transparency of biological tissues to the emission of these dyes makes them attractive for biological imaging.

The purpose of this communication is to demonstrate the use of tunable fiber laser excitation for two-photon imaging. The method has the potential to enhance imaging capabilities of two-photon microscopy by the availability of multiple excitation wavelengths in a readily tunable and portable fiber source.

2. Solitonic wavelength shifting

For the first order soliton produced in a PCF, the peak power, P, the chromatic dispersion of the fiber, D, pulse width, T_0, and the nonlinearity of the fiber, γ, are related by [12]:

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$$\frac{2\pi c\gamma P T_0^2}{\lambda^2 |D(\lambda)|} = 1, \qquad (1)$$

where $D > 0$ (anomalous dispersion) is required. Since the chromatic dispersion, in general, increases with wavelength, the balance between fiber nonlinearity and chromatic dispersion moves toward longer wavelength with increasing optical power. Therefore, the wavelength of the optical soliton can be changed by adjusting the power launched into the fiber (7). This fact offers the potential in future development for rapid wavelength switching by modulation of the power into the fiber, for example with an acousto-optic modulator. Conventional optical fibers made for optical communications usually have zero dispersion wavelengths around 1300 nm with positive dispersion slope, and therefore the chromatic dispersion in the 800 to 1300 nm region is negative. In this case, an optical soliton will only be produced for excitation wavelengths above 1300 nm. In contrast, PCF's can be designed with a higher level of flexibility. The zero dispersion wavelengths of a PCF can be made as low as 650 nm and the nonlinear coefficient, γ, can be made two to three magnitudes higher than conventional fibers, making it possible to efficiently shift the wavelength of a soliton [13].

To verify nonlinear PCF wavelength shifting in the 800 nm wavelength region, we have recorded the spectrum of the output from a ~6-m PCF (NL-1.8-710, Thor Labs Inc., Newton, NJ). The dispersion and dispersion slope for this fiber are 68 ps/nm/km and 0.59 ps/nm^2/km, respectively, at 800 nm, and 148 ps/nm/km and 0.25 ps/nm^2/km at 1000 nm. The core diameter is 1.8 ± 0.1 μm mode field diameter is 1.1 ± 0.1 μm at 710 nm. (Further specifications for the fiber are available from the manufacturer.) The excitation source was a 780 nm fiber laser operating at 20 mW average optical power, 100 fs pulsewidth, 50 MHz repetition rate (Femtolite Ultra, IMRA America, Inc., Ann Arbor, MI). The excitation laser beam was coupled into the PCF through a 20x objective (Newport Corp., Irvine, CA). For this initial demonstration of wavelength tuning, the optical power coupled into the fiber was adjusted by simply moving the tip of the fiber axially into and out of the focus of the coupling objective. A spectrum analyzer (Ando AQ-6315B) was used to record the spectrum.

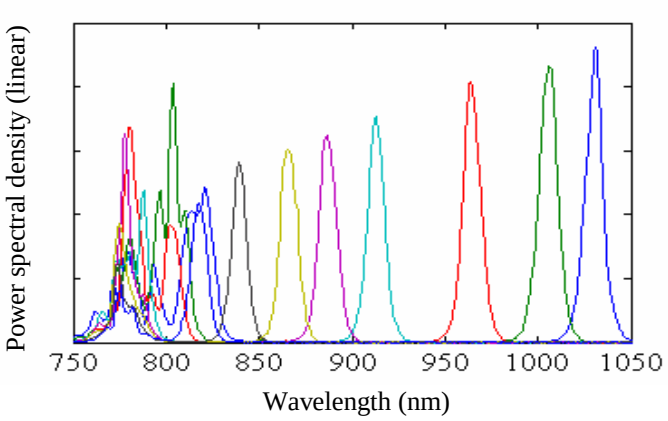

Fig. 1. Example of measured soliton wavelength shift by adjusting coupled power levels from a 20mW fiber laser into a 6 m PCF.

Figure 1 demonstrates wavelength shifting from 820 nm to >1035 nm as a function of coupled laser power in the PCF. The output optical power was approximately 4 mW at the shifted wavelength of 1035nm. Output pulse durations are expected to be broadened only slightly due to the mechanism of the soliton self-frequency shift [7]. In our experiment, the input power was adjusted simply by defocusing at the fiber tip, and we were not able to measure exactly how much power that was actually coupled into the PCF. Detailed experimental and theoretical descriptions on wavelength shift versus input power change can

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be found in a number previous works [7, 13]. As Fig.1 shows, the pulse optical power increases linearly with the wavelength because of the increased coupled optical power into the fiber. For example, the estimated average power at 825 nm is 2.1 mW. Approximately 40% of the output optical power remained in the original wavelength and was not converted into a fiber soliton because of the pulse shape mismatch of the laser source. In addition, when the fundamental soliton wavelength is longer than 1040 nm, a second order soliton starts to appear at the wavelength approximately 200 nm shorter than the fundamental soliton. The condition for the creation of a second-order soliton is an input peak power four times the power of the first order soliton [12], so the second-order soliton can be avoided with an optical power below this threshold, which depends on the design of the fiber. The wavelength separation between the first and the second order solitons also depends on the dispersion slope of the fiber. With proper design and optimization of the PCF, this separation can be maximized in the future. In any case, these unwanted wavelength components can be easily removed by using proper long-pass filters.

Since propagation of the optical soliton is based on the balance between nonlinearity and chromatic dispersion, a fiber with a lower nonlinear parameter would require a higher optical power to maintain the soliton condition if the dispersion characteristic is not changed. Therefore if the PCF can be optimized to have a larger core-diameter and or higher anomalous dispersion value, in principle, it should be possible to generate higher powers in shifted wavelengths providing that the fiber laser is powerful enough. Based on 4 mW at 1035 nm generated with the 20-mW fiber laser, we expect to be able to generate 20 mW at 1035 nm using a 100-mW fiber laser and optimized PCF, decreasing roughly linearly to 10 mW at 825 nm. These powers are sufficient for many two-photon imaging applications [4, 14].

3. Multicolor two-photon excitation in cells

In order to demonstrate the application of two-photon wavelength scanning to biological imaging, we imaged a multiply labeled standard cell bovine pulmonary artery endothelial cells (BPAE) (Fluocells Slide #1, Molecular Probes, Eugene, OR). For this application, ultrafast pulses were generated at 810 nm by a 75-MHz, 100-mW fiber laser with pulse widths of approximately 100 fs (Femtolite F100, IMRA America, Inc., Ann Arbor, MI). In order to adjust the amount of wavelength shifting, the laser power coupled into the PCF was attenuated either by a variable neutral density filter or by defocusing at the coupling point as described above. For excitation wavelengths shorter than 1000 nm, no second order solution is produced by the PCF and an 830 nm long pass color glass filter (Schott RG830) was used to reject the remnant light at the original wavelength of the fiber laser. For PCF output wavelengths longer than 1000 nm, a custom 975 nm long pass filter (d1100/250x, Chroma Tech. Corp., Rockingham, VT) was used which to reject the second order soliton and other short wavelength components created in the PCF. Cells were imaged with a fluorescence scanning-stage microscope (Nikon TE2000) with a 100x 1.3 NA Superfluor objective lens and data collection system similar to that described previously [15]. The sample was raster scanned across the objective by a piezoelectric scanning stage (Mad City Labs, Nano H100). Images were obtained with a step size of 250 nm, pixel dwell time of 2.5 ms, and average powers of 600 to 800 μW into the microscope objective. In the present case, a 700-nm short pass dichroic filter (uvnirpc2, Chroma) was used to reflect the laser light to the sample and pass the fluorescence to the detector. A custom 680 nm short pass filter was used to filter out the excitation light at the detector (e680sp-2p, Chroma).

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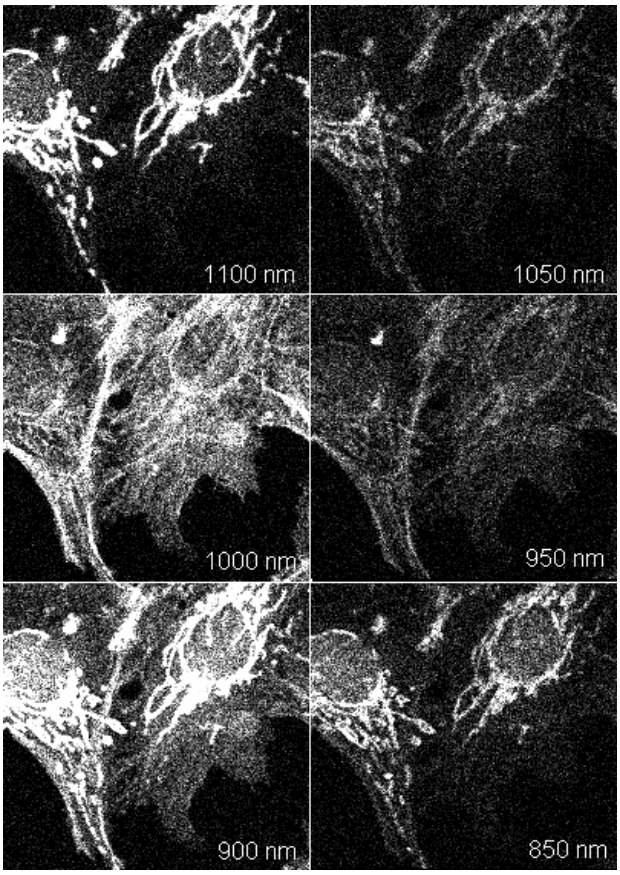

Fig. 2. Two-photon fluorescence images of Fluocells® slide #1 with two-photon excitation wavelengths from 850 nm to 1100 nm. Average power at the back of the objective was approximately 800 μW at 1000 nm 800 μW at 1000 nm.

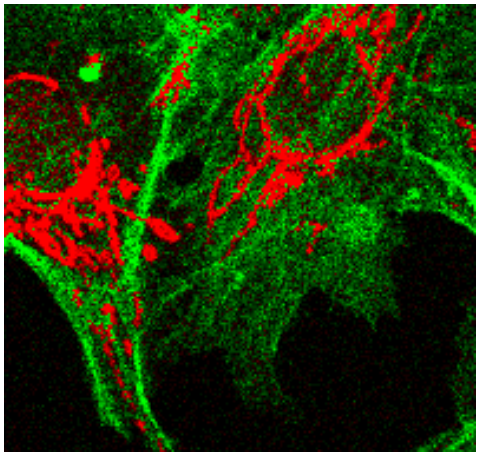

Fig. 3. A. False color image combining the scans at 1100 nm (red) and 1000 nm (green) from Fig.2 showing selective excitation of F-actin filaments stained with Bodipy-Fl at 1000 nm and of mitochondria stained with MitoTracker Red at 1100 nm

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Two-photon images of BPAE cells are shown in Fig.2. The mitochondria were stained with MitoTracker Red (a xanthene dye derivative) and the F-actin filaments were stained with Bodipy-Fl. Fig.2 demonstrates clear selective excitation of Bodipy-Fl at 1000 nm and of MitoTracker Red at 1100 nm. Fig.3 displays a false-color image created from the 1000 nm and 1100 nm images demonstrating selective imaging of the F-actin and mitochondrial structures targeted by Bodipy-Fl and Mitotracker Red, respectively. These images demonstrate the selective two-photon excitation of Bodipy-Fl and MitoTracker Red. The ratio of Bodipy-Fl relative to Mitotracker Red fluorescence intensities as a function of two-photon excitation wavelength is plotted in Fig.4. These intensities were selected from areas of the image that contained high concentrations of Bodipy-Fl and Mitotracker Red, respectively.

While the possibility of selective two-photon excitation has been demonstrated previously for fluorescent proteins [9], it has not been clear for smaller organic dyes. A key to selective excitation for Bodipy-Fl and Mitotracker Red was the ability to excite at wavelengths out to 1100 nm. Comparison of the images at 1050 nm and 1100 nm (Fig. 2) shows that Mitotracker Red was excited much more efficiently at 1100 nm than at 1050 nm. This fluorophore has a maximal excitation wavelength in the visible at 580 nm. Thus two-photon excitation at 1100 nm likely occurs via the well known vibronic band of xanthene dyes (such as fluorescein and tetramethylrhodamine). It has been shown that this band is enhanced in the two-photon excitation spectrum of fluorescein relative to the origin band compared to its relative excitation probability by one-photon excitation [11]. It is interesting to note that there are many dyes with their maximum absorption for the lowest excited electronic state in a wavelength range similar to that of Bodipy-Fl (450 to 500 nm) that can be two-photon excited at 900 to 1000 nm. In addition Mitotracker red is similar spectrally and structurally to several xanthene dyes (e.g. Texas Red) that may also have minima in their excitation spectra in the 1000 nm region. Thus it seems likely that many dye combinations could be selectively excited by wavelength switching between 1000 nm and 1100 nm, similarly to Bodipy-Fl and MitoTrackerRed since 1100 nm is too low in energy to excite these fluorophores via the two-photon process. Wavelength-switching TP-FLEX therefore holds promise for new approaches in two-photon microscopy.

We note however that the excitation power increases with wavelength as a result of the power dependence of the soliton frequency shift as shown in Fig. 1. This wavelength-dependent power variation is deterministic and is approximately linear; therefore it can be compensated at the PCF output by a specially designed compensating optical filter in which the transmission efficiency is linearly reduced versus wavelength.

4. Conclusion

We used a fiber laser in combination with a PCF for simple excitation wavelength scanning for two-photon imaging. Although we have generated images with a scanning stage system, optimization of the PCF for increased power conversion would potentially allow an increase in scanning speed for applications of two-photon laser scanning microscopy. Further development of TP-FLEX is planned by implementation of rapid wavelength tunability through modulation of the input power into the PCF, and by optimization of the PCF design to increase the power conversion into the first-order soliton. We also demonstrated selective two-photon photoexcitation of the fluorophores Bodipy-Fl at 1000 nm and Mitotracker Red at 1100 nm. The ability to access wavelengths above 1000 nm in a continuously tunable way with a small, turnkey fiber based laser source presents the potential for wavelength switchable two-photon imaging. Further, a fiber laser source with PCF would be readily portable from one microscope to another, rather than being fixed to one system. It needs to be pointed out that so far, the major limitation of wavelength tuning using PCF is the low optical power at the PCF output. This is mainly limited by the characteristics of the available PCFs and the peak output optical power of the fiber laser. With current rapid advances of fiber laser technology and the optimization of PCF characteristics for this purpose, the output optical power should reach a significantly higher level.

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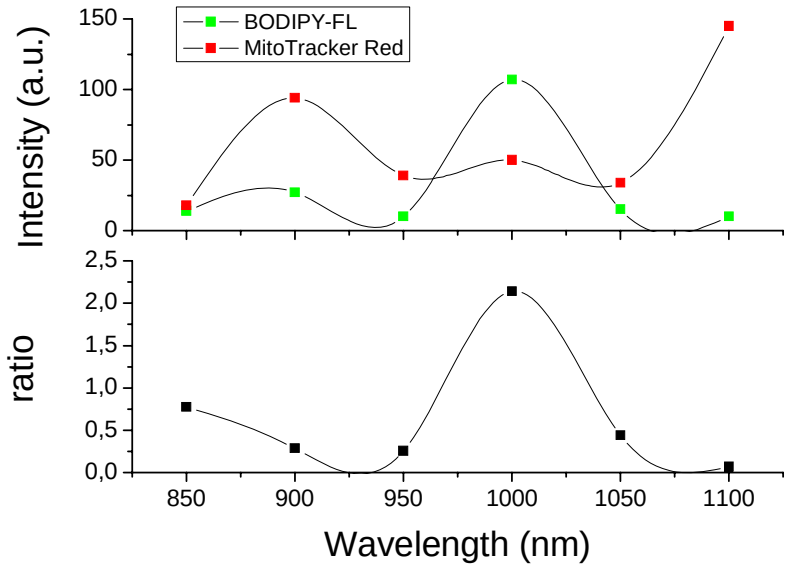

Fig. 4. Top: Relative two-photon excitation intensities for Bodipy-Fl and MitoTracker Red computed from areas where these species predominate in the images in Fig.2. Intensities were not corrected for the dependence of excitation power on wavelength (see Fig.1). Bottom: Ratio of fluorescence intensities of Bodipy-Fl intensity to Mitotracker Red over the range of excitation wavelengths shown in Fig.2. Lines are included merely to guide the eye.

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