Single-Fiber Optical Probe for Two-Photon Induced Fluorescence of Biological Markers

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The use of fluorescent dyes which can be attached to specific cellular components as biological markers has become a well-established biomedical technique in recent years [1]. When optically stimulated, these dyes emit a characteristic fluorescence, enabling the structure and organization of the stained sample to be readily studied and visualized by means of fluorescence spectroscopy and confocal microscopy [2]. Moreover, some dyes exhibit enhanced fluorescence if attached to certain biological molecules. For instance, 4',6-diamidino-2-phenylindole (DAPI) exhibits such enhanced fluorescence when attached to the AT base pairs on a DNA strand [3]. Such properties are useful for mapping DNA structure or identifying the presence of particular molecules in a sample.

Typical dyes used in biological studies absorb and emit visible or UV light. However, UV light is strongly absorbed within a thin layer near the surface of the sample. Other problems with UV pumping include the lack of inexpensive UV laser sources, photobleaching of the dye molecules, and the anticipated photodamage of living cells by energetic UV photons. However, with the advent of ultrashort laser pulses, a new technique has emerged to excite these dyes with visible light which overcomes many of the aforementioned problems. This technique employs two-photon absorption in the dye, a third-order nonlinear process whereby a dye molecule absorbs two long-wavelength photons instead of a single short-wavelength photon. Subsequently, the dye emits its characteristic fluorescence and returns to its ground state. The whole process is referred to as two-photon induced fluorescence (TPIF) [4].

TPIF output intensity depends quadratically on the *peak* intensity of the pump laser pulse. Therefore, it is possible to observe TPIF with a modest *average* power if the laser pulse is sufficiently short. A major advantage of TPIF over single-photon induced fluorescence is potentially higher resolution. This is a direct consequence of the fact that TPIF is observed only in the immediate vicinity of the focal spot, due to its quadratic intensity dependence. TPIF has already been proven useful in confocal laser scanning microscopy [5].

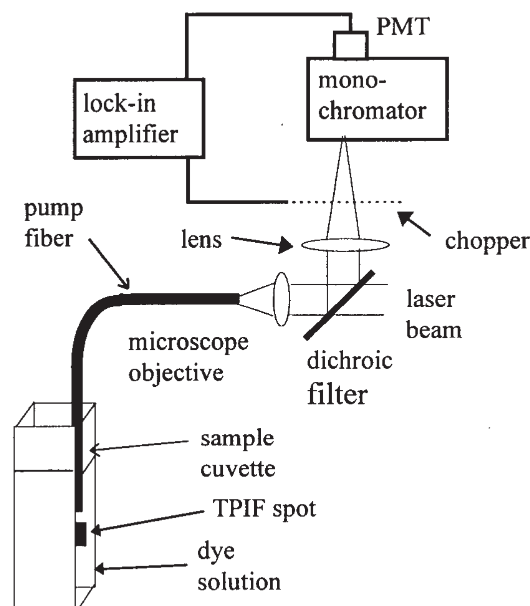

Fig. 1 The experimental setup.

However, TPIF encounters its own problems, the most obvious being its apparent inability to probe the *inhomogeneous* media widely present in biological research. Such media severely scatter incident laser light, thus preventing the tight focusing needed to observe TPIF, and seemingly limiting TPIF applications to investigating layers near the surface. It has already been shown that this shortcoming can be overcome by using optical fibers to deliver the pump light into the inhomogeneous medium [6]. Moreover, TPIF with fibers can be extended to biological media of very low transparency.

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Optical fiber sensors are widely used in a variety of fields [7]. In experiments with TPIF, optical fibers offer enhanced resolution due to the very small spot sizes achievable within the specimen. Therefore, a smaller volume is exposed to laser intensity high enough to observe TPIF, which also limits the size of the region that could be damaged or photobleached. Moreover, the use of laser sources in the red part of the spectrum allows us to utilize off-the-shelf optical fibers instead of special expensive fibers required for UV radiation. In this paper we demonstrate that a single optical fiber can be successfully used to induce TPIF and collect the fluorescence to perform spectroscopic studies in liquid dye solutions. We also compare single-mode and multimode fibers and show that single mode fibers have the advantage for high resolution application.

The laser system used in our experiments consists of a Coherent 700 series dye laser pumped with the second harmonic of a mode-locked Coherent Nd:YAG Antares laser. The dye laser utilizes Pyridine as a gain medium with a saturable absorber, and a cavity dumper to shorten the pulses and to control the repetition rate. At a repetition rate of 7.6 MHz, the Pyridine laser produces 2 ps pulses at 730 nm with an average power of 70 mW; this corresponds to a peak power of 5 kW.

Five different optical fibers were used to deliver the laser light into the solution as indicated in table 1. The fibers were purchased from Thor Labs. The f_5_12 fiber supported only a single spatial mode, while the other four supported multiple modes as observed with a CCD camera. (The first number in a fiber's code is the core diameter in units of mm while the second number is the numerical aperture X 100).

Fiber	Core Diameter (μm)	N.A.	Coupling Efficiency (%)
f_5_12	5.5	0.12	10
f_5_12_10X	5.5	0.12	20
f_50_37	50	0.37	80
f_100_37	100	0.37	67
f_125_48	125	0.48	67
f_200_48	200	0.48	72

Table 1. Properties of the five fibers.

The laser beam was injected into the fibers with a 5X microscope objective one fiber at a time (except for one case where a 10X lens was used as indicated). We used a variable neutral density filter to change the input laser power. The output ends of the fibers were immersed in a dye solution contained within a standard fluorometer glass cuvette. The back-scattered sample fluorescence was collected from the pump fiber with a dichroic filter as shown in figure 1. It should be noted that a chopper placed in front of the monochromator was used with a lock-in amplifier to enhance the fluorescence signal.

The experiment was performed with a 5 mM solution of DAPI in Methanol. Figure 2 shows the one of the acquired spectra.

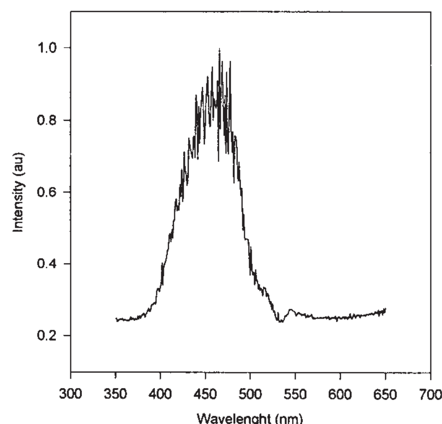

Figure 2. DAPI fluorescence spectrum.

The fluorescence spectra acquired at several values of input laser power were integrated over the entire fluorescence band to obtain the integrated intensity of TPIF. By varying the input power and computing the integrated fluorescence, we show in Fig. 3 the quadratic dependence of the fluorescence on the exiting power for the fibers we used.

Our previous work [6] revealed that the smaller core diameter of a fiber is, the more efficient a pumping source it is. As the core diameter increases, the generated fluorescence signal drops. The difference is due to the spatial profile of the beam exiting the fiber. The smaller the fiber is, the more tightly focused the beam is at the exit. Since the generated

fluorescence is quadratically proportional to the pump intensity, a higher fluorescence signal is generated with smaller fibers. Moreover, the exiting beam diverges more quickly for smaller fibers. Hence, the smaller the fiber, the more

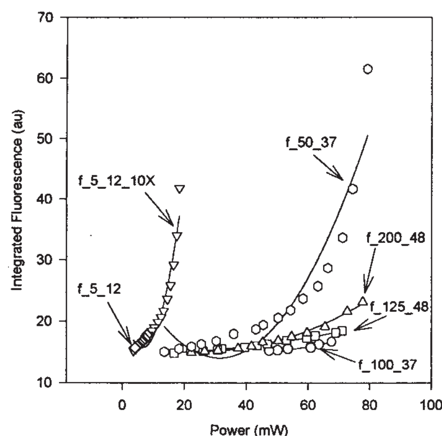

Fig. 3 Quadratic dependence of fluorescence on pump power for the different fibers.

intense and spatially confined its fluorescence. We have observed, with the aid of a CCD camera, that the fluorescence is more concentrated near the tip of smaller fibers. This trend was clearly observed as we compared the f_5_12 fiber with the f_50_37 fiber and was further verified by studying the f_100_37 fiber as well as the two other higher.

In this experiments, we collected the back scattered fluorescence signal with the aid of a dichroic filter. We had previously speculated that Multimode fibers can be more efficient at collecting the fluorescence. However, it is evident from figure 3 that this is not the case. The single mode fiber, despite its low coupling efficiency, proved to be best suited for the task. This somewhat surprising result is due to two factors. First, the fluorescence profile is fairly well matched to the numerical aperture of any given fiber. This is a direct result of the quadratic dependence of fluorescence on the pump intensity which serves to produce a tight fluorescence profile. Second, the single-mode fiber produces the highest intensity at its tip due to its narrow core diameter. This results in a strong fluorescence signal part of which is coupled efficiently back into the fiber and collected by our detection optics.

This arrangement has many useful applications in chemical, environmental and biological sensing. A single-fiber optical probe can be constructed to test for the presence of chemicals in various environments. Moreover, the small spot produced by the single mode fiber would ideal for TPIF fiber microscopy where a new class of samples can be accessed and imaged.

In conclusion, we have demonstrated the feasibility of using optical fibers to produce and detect two-photon induced fluorescence of two different biological markers. This technique is applicable to a variety of dyes important for biological research. It is shown that due to stronger fluorescence confinement near the fiber tip, single mode fibers are more suitable for high resolution applications.

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