Two-photon-induced fluorescence of biological markers based on optical fibers

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Optical fibers were used to induce fluorescence by means of two-photon absorption in fluorophores. The effect, studied in a solution of 4′,6-diamidino-2-phenylindole, demonstrated that a single-mode fiber is a more efficient two-photon excitation source than a multimode fiber. This was shown with three different fibers. © 1995 Optical Society of America

The use of fluorescent dyes that 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, permitting 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 that overcomes many of the aforementioned problems. This technique employes 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 as a result of its quadratic intensity dependence. TPIF has already been proven useful in confocal laser scanning microscopy.[5] 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 for observation of TPIF and seemingly limiting TPIF applications to investigating layers near the surface. We believe that this shortcoming can be overcome with optical fibers to deliver the pump light into the inhomogeneous medium and to extract the TPIF signal. Moreover, TPIF with fibers can be extended to biological media of very low transparency.

Optical fiber sensors are widely used in a variety of fields.[6] In experiments with TPIF, optical fibers offer enhanced resolution because of the very small spot sizes achievable within the specimen. Therefore a smaller volume is exposed to laser intensity high enough for observation of 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 permits us to utilize off-the-shelf optical fibers instead of special expensive fibers required for UV radiation. In this Letter[7] we demonstrate that optical fibers can be successfully used for TPIF spectroscopy in liquid dye solutions, especially in the presence of strong scattering. 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 uses 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.

Three different optical fibers, listed in Table 1, were used to deliver the laser light into the solution. The fibers were purchased from Thor Laboratories. The f_5_12 fiber supported only a single spatial mode,

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Table 1. Properties of the Three Fibers

Fiber[a]	Core Diameter (μm)	N.A.	Coupling Efficiency (%)
f_5_12	5.5	0.12	20
f_50_37	50	0.37	70
f_100_37	100	0.37	73

[a]The first number is the core diameter, and the second number is the N.A. times 100.

while the other two supported multiple modes, as observed with a CCD camera. (The first number in a fiber's code is the core diameter in units of micrometers, and the second number is the numerical aperture times 100.) The laser beam was injected into the fibers with a 10× microscope objective, one fiber at a time. The respective coupling efficiencies (incident to the output power ratio) are listed in Table 1. 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 sample fluorescence was collected at a 90° angle relative to the direction of the pump beam. A glass image conduit with a 1.5-mm diameter gathered the light and delivered it to the monochromator.

The experiment was performed with a 5 mM solution of DAPI in methanol. The fluorescence spectra acquired at several values of input laser power were integrated over the entire fluorescence band to yield the integrated intensity of TPIF. Figure 1 shows the experimental setup and the fluorescence spectrum of DAPI. By varying the input power and computing the integrated fluorescence we show in Fig. 2 the quadratic dependence of the fluorescence on the output power for the fibers that we used. The figure reveals that the smaller the core diameter of a fiber, the more efficient a pumping source it is. As the core diameter increases, the detected fluorescence signal drops. The difference is due to the spatial profile of the beam leaving the fiber. The smaller the fiber, the more tightly focused the beam is at the exit. Since the detected fluorescence is quadratically proportional to the pump intensity, a higher fluorescence signal is detected from smaller fibers. Moreover, the output beam diverges more quickly for small fibers. Hence the smaller the fiber, the more intense and spatially confined its fluorescence. We have observed with the aid of a CCD camera that the fluorescence is more concentrated near the tips of smaller fibers. This trend was clearly observed when we compared the f_5_12 fiber with the f_50_37 fiber and was further verified by study of the f_100_37 fiber as well as two other higher-N.A. fibers not listed in Table 1.

In the second part of our experiment we investigated the use of optical fibers in the presence of scatterers in the solution. The scatterer that we used consisted of a 70% solution of silica particles in water. This solution, called Ludox, was obtained from Dupont. A Ludox drop of 10-μL volume was added to our solution of total volume of 1.12 mL. We repeated the experiments and plotted the results in Fig. 3. As expected, the fluorescence signal dropped with the addition of scatterers to the solution for all fibers except the f_5_12 fiber. Interestingly, the fluorescence signal did not change when the experiment was per-

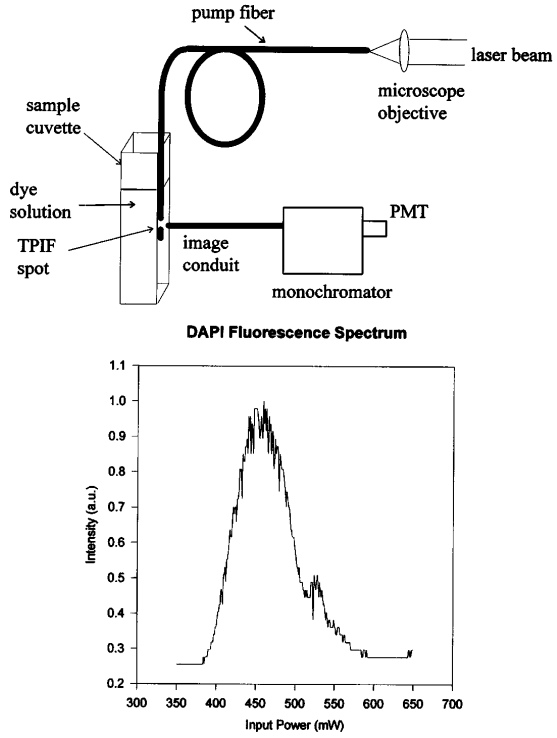

Fig. 1. Geometry used to deliver the laser light and collect the fluorescence signal. The dye-laser beam is coupled into the pump fiber and a 10× microscope objective, the fiber tip is immersed in the dye solution close to the wall of the cuvette, and then the image conduit collects the light on the other side of the cuvette wall and delivers it to the monochromator. PMT, photomultiplier tube. The detected spectrum of DAPI is also shown.

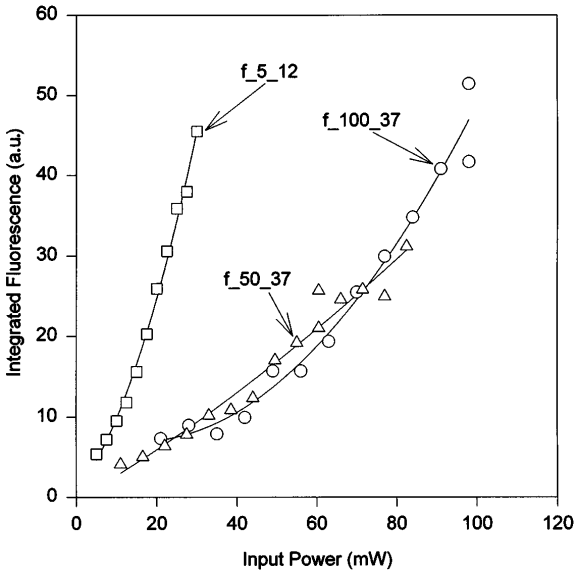

Fig. 2. Fluorescence intensity versus pump intensity obtained for the different optical fibers. The solid curves are quadratic fits to the data. Small linear and constant terms exist in the fitted curve owing to nonideal fundamental wavelength suppression in our monochromator.

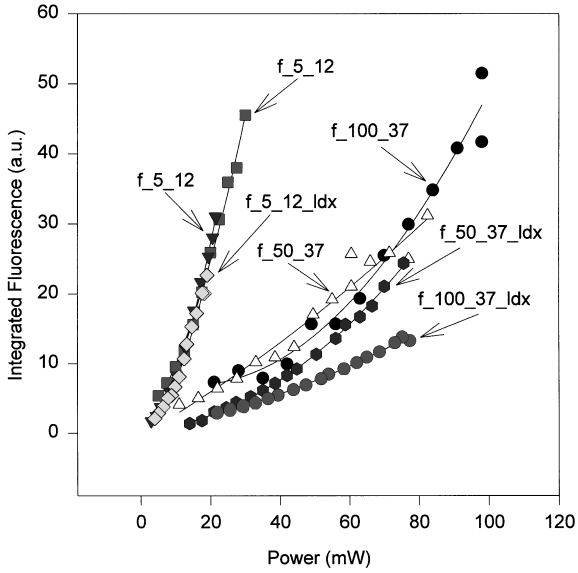

Fig. 3. Fluorescence intensity versus pump intensity in the presence of scatterers. The solid curves are quadratic fits to the data. The fluorescence signal decreases for all but the smallest fiber. The ldx suffix refers to the presence of the scatterer Ludox.

formed with the f_5_12 fiber. We explain this by the fact that the beam is focused to a small spot in the dye where two-photon absorption takes place, and the likelihood of finding a scatterer is very small. We confirm this by noting that the larger the core diameter of a fiber was, the more the fluorescence signal dropped in the presence of scatterers. Our results clearly indicate that a single-mode fiber is best suited for addressing a small volume of dye and generating a large signal.

In our experiments we collected the fluorescence signal with an image conduit. The next step will be to collect the signal with the same (pump) fiber by use of a dichroic beam splitter or an off-axis parabolic mirror, or with a second fiber at an angle to the pump fiber. Multimode fibers can be more efficient at collecting the fluorescence. The arrangement in which the pump fiber collects the backward fluorescence can be used for TPIF fiber microscopy, which would require the use of high-N.A. fibers that can collect the backscattered fluorescence more efficiently.

In conclusion, we have demonstrated the feasibility of using optical fibers to study 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, owing to stronger fluorescence confinement near the fiber tip, single-mode fibers are more suitable for high-resolution applications.

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