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SINGLE-FIBER TWO-PHOTON FLUOROPROBE FOR BIOLOGICAL MARKERS

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We demonstrate the feasibility of using a single optical fiber to simultaneously excite and collect two-photon-induced fluorescence in fluorophores. We show that single-mode fibers are more efficient as a source and detector than available multimode fibers.

The fluorescence emission of biological markers has long been used to probe cellular environments with a variety of techniques such as fluorescence spectroscopy, confocal microscopy, and correlation spectroscopy. These markers are typically excited with UV light and their fluorescence emission is detected and analyzed for information. A new technique is emerging where the fluorophores are excited through the process of two-photon absorption (TPA). This is achieved by using ultrashort laser pulses that possess the high peak powers necessary for TPA. This process of two-photon-induced fluorescence (TPIF) is increasingly being applied to the various fields of biological fluorescence spectroscopy.[1–4] TPIF can be applied to the study of autofluorescence (naturally-occuring fluorescence) in various molecules such as proteins where it can reveal information about their structure. Hence, from the point of view of these applications, it would be greatly advantageous to generate TPIF with an optical fiber. Furthermore, it would be ideal to be able to collect the fluorescence with the same fiber. In this paper, we demonstrate, for the first time to the best of our knowledge, the feasibility of a single-fiber probe.

In a previous study,[2] we demonstrated that optical fibers can be used efficiently for TPIF and offer many advantages. Among these advantages is the ability to access turbid and scattering environments, such as blood, which may not be accessible with conventional TPIF techniques. This is achieved by using an optical fiber to deliver high intensity light capable of generating TPIF in a small volume. This small illumination volume also holds promise for fiber TPIF scanning microscopy.

The study[2] showed that smaller, single-mode, optical fibers are more efficient pump sources than larger multimode fibers. At that time, the fluorescence was collected with an image conduit at a 90° angle to the pump fiber. However, it is clear that for most practical purposes, it would be beneficial to combine these two channels (excitation and detection) into one by using a single fiber. The advantages of such integration are obvious. Such a system is capable of accessing scattering and turbid media where light delivery and detection may not be possible by any other means. Moreover, given the small volume of the generated TPIF spot and the ability to spatially scan the fiber tip, one can conceive of a variety of scanning microscopy schemes including nonlinear near-field scanning fluorescence microscopy.

In this paper, we report on developing a single-fiber fluoroprobe that is capable of producing TPIF *and* capturing the resulting fluorescence for analysis. We compared single-mode and multimode fibers to ascertain which fiber is best suited for the task. This comparison is needed since it is not obvious that single-mode fibers would perform better in this configuration. Our previous work showed that single-mode fibers are better excitation sources since they produce a smaller illumination spot. However, detection requires a large numerical aperture. Thus, it is clear that there must be an optimal balance between the fiber radius to produce a single mode and its numerical aperture. Our simple theoretical analysis shows the existence of just such an optimal core radius for the fiber for a given value of numerical aperture.

The experiment was performed in a 5 mM solution of 4',6-diamidino-2-phynelindole (DAPI).[5] DAPI is a common fluorescent probe that exhibits enhanced fluorescence when attached to the AT base pairs on a DNA strand. The laser system we used consisted of a Coherent 700-series dye laser pumped with the second harmonic of a mode-locked Coherent Nd:YAG Antares laser. The dye laser utilized Pyridine as a gain medium with a saturable absorber and a cavity dumper. The pulses had a repetition rate of 7.6 MHz, a width of 2 ps, and an average power of 100 mW at 730 nm. This corresponds to a peak power of $\sim$ 5 kW. It should be noted here that sub-picosecond pulses can be used to achieve similar results at lower average powers. However, due to their typically higher peak intensity, one incurs the risk of damaging the optical fiber. Moreover, for time-resolved experiments, sub-picosecond pulses would suffer more broadening in the fiber than picosecond pulses.

Figure 1 shows the rest of our experimental setup. The laser pulses pass through a dichroic filter to a microscope objective that injects the light into the fiber. The tip of the fiber is immersed in a cuvette that contains the DAPI solution. As light emerges from the fiber, the intensity is high enough near the tip to generate TPIF. The fluorescence is visible to the unaided eye in ambient lighting. Some of the fluorescence is back-scattered into the fiber. This back-scattered fluorescence is reflected by the dichroic filter to a gathering lens that focuses the light through a chopper onto a monochromator. The PMT signal is fed to a lock-in amplifier for signal detection to achieve high detection levels.

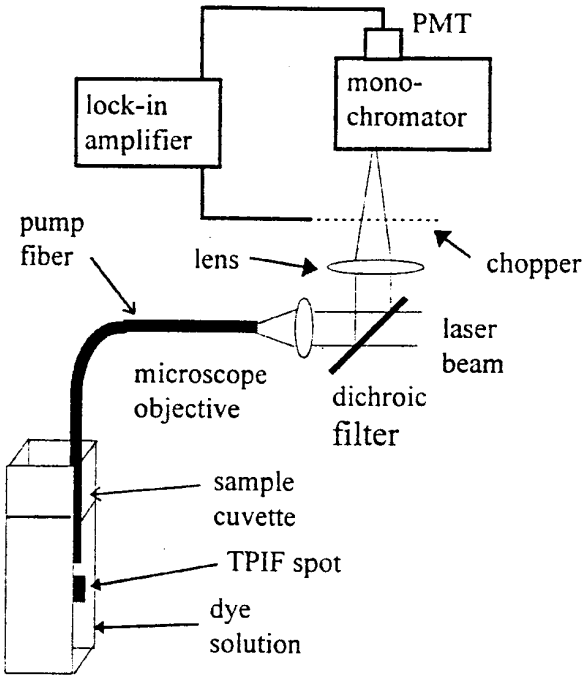

Fig. 1. The experimental setup.

One single-mode and two multimode fibers were tested. These fibers and their properties are listed in Table 1. The first number in the fiber code is its core diameter in units of μm and the second number is its numerical aperture multiplied by 100. The coupling efficiency varied depending on the core diameter and the numerical aperture. A 5X microscope objective was used except in one case where a 10X objective provided better injection efficiency. This is indicated with 10X suffix.

Table 1. Fiber parameters.

Fiber[a]	Core diameter (μm)	N.A.	Coupling efficiency (%)
f_5_12	5.5	0.12	10
f_5_12_10X[b]	5.5	0.12	20
f_100_37	100	0.37	67
f_125_48	125	0.48	67

[a]The first number is the core diameter in μm, and the second number is the NA $\times$ 100.
[b]10X objective was used instead of 5X objective

The fluorescence was successfully generated and captured using our experimental setup (A typical fluorescence spectrum can be found in Ref. 2). The input power into the fiber was adjusted using a variable neutral density filter. The signal from each fiber was recorded for a wide range of input powers. The resulting fluorescence

spectrum was integrated over the wavelength to produce a single value for a given input power. The result is shown in Fig. 2. The horizontal axis indicates the amount of optical power exiting the fiber into the dye solution. The solid lines are quadratic fits to data. They confirm the quadratic dependence of the spectrum on the input power. The experimental points do not appear to return to the origin as theory would suggest. This is due to the fact that our setup is not noise-free and there is a residual background signal that leaks into the monochromator and produces the offset. It should also be noted that the experiment can be performed with much lower concentrations by keeping a high input intensity.

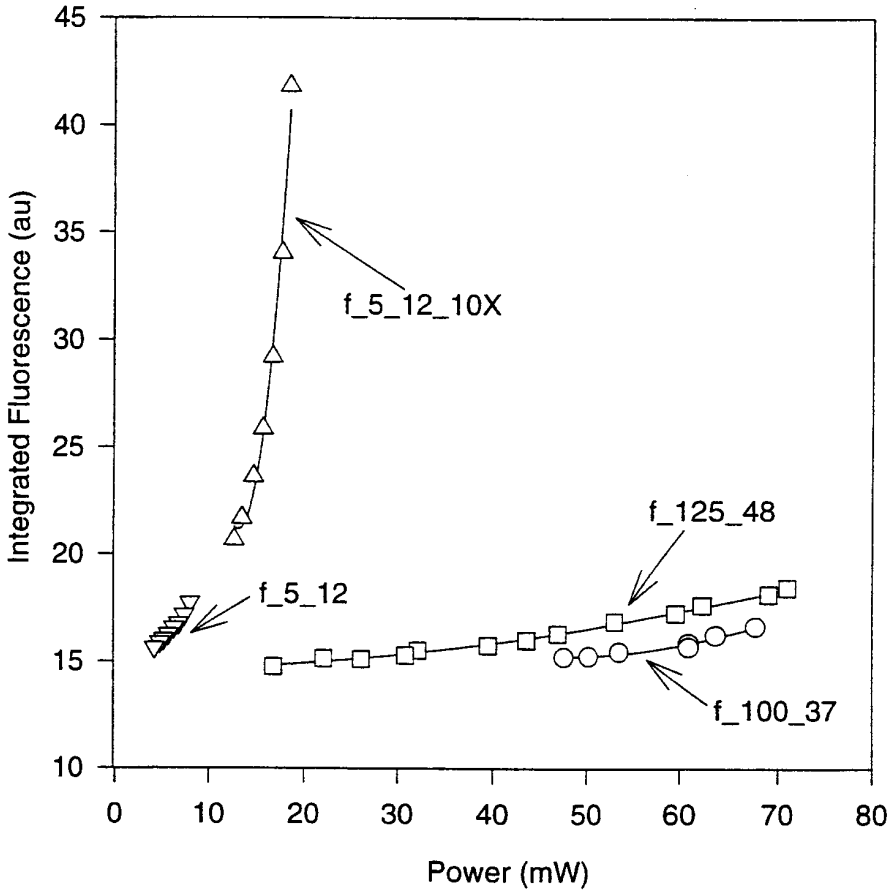

Fig. 2. Fluorescence intensity as a function of pump intensity. The solid curves clearly indicate the quadratic dependence on intensity. The curves are marked by the codes appearing in Table 1.

As discussed above, our previous work[2] indicated that fibers with smaller core diameters and smaller numerical apertures are more efficient pump sources. In trying to combine both pumping and collection functions in one fiber, one expects an optimum balance between a fiber's core radius and its numerical aperture. It is not obvious that this balance would not occur in a multimode (i.e. large diameter)

rather than single-mode fiber. However, we found in our experiments that the single-mode fiber overall efficiency exceeded that of the two multimode fibers. The observed effect is greatly beneficial for most TPIF applications.

Our theoretical discussion develops a model for the single-mode fiber since our experimental results show that it is of greater interest. Note that the quadratic dependence of TPIF on the pumping intensity at each spatial point results in a random distribution of "hot spots" of TPIF. These hot spots are random in location, size, and intensity. Indeed, we observed such spots by imaging the multimode fiber with a CCD camera. The theory of TPIF due to scattering will be addressed by us elsewhere.[7] However, it is obvious from our experiments that regardless of the exact nature of the effect, the efficiency of back-scattered TPIF from multimode-originated hot spots is significantly lower than from the well-defined and highly-concentrated pumping field originated by a single-mode fiber. Thus, we concentrate here on the more relevant latter case to obtain an estimate for the single-mode fiber efficiency.

We start by assuming that the pumping intensity distribution is well-approximated by a fundamental Gaussian beam,[8]

$$I(z) = \frac{2P_i}{\pi w^2(z)} \exp(-2r^2/w^2(z)), \tag{1a}$$

$$w^2(z) = w_0[1 + (z/z_0)^2]^{1/2}, \tag{1b}$$

$$z_0 = \frac{\lambda}{\pi\theta_0^2} = \frac{\pi w_0^2}{\lambda}, \tag{1c}$$

$$w_0 = [0.65 + 1.619V^{-3/2} + 2.87V^{-6}]a, \tag{1d}$$

$$V = \frac{2\pi a}{\lambda} NA, \tag{1e}$$

$$\theta_0 \sim \sin^{-1}(NA), \tag{1f}$$

$$NA = \frac{(n_{\text{core}}^2 - n_{\text{clad}}^2)^{\frac{1}{2}}}{n_{\text{medium}}}, \tag{1g}$$

where P_i is the power of the input (pump) beam, λ is the wavelength of the pump beam, and n is the index of refraction. We shall first estimate the power in the fluorescence spot. This is done by assuming an overall pumping and absorption efficiency factor β, and integrating radially to find the differential power contained in a differential slice in the z-direction;

$$dP_F(z) = \int_0^{2\pi} d\phi \int_0^{\infty} r dr \beta I^2(z) dz = \frac{\beta P_i^2}{\pi w^2(z)} dz. \tag{2}$$

We assume that the fluorescence spot has a Lambertian (dipole) radiation pattern which has the form $\cos(\theta)$ where θ is the angle from the propagation axis. This

choice of pattern is consistent with the fact that the fiber collects light from a small angle even though the fluorescence is actually isotropic. The coupling efficiency of a Lambertian source into a fiber[9] is $\eta = A_f/A_z NA^2$ where A_f is the fiber core area, πa^2, and A_z is the area of the differential fluorescence element, $\pi w^2(z)$. Therefore, the differential returned power is

$$dP_r(z) \sim \eta dP_F(z) \sim \frac{\beta P_i^2 NA^2 a^2}{\pi w^4(z)}, \tag{3}$$

and the total returned power is

$$P_r \sim \int_0^\infty \frac{\beta P_i^2 NA^2 a^2}{\pi w_0^4} \frac{1}{[1+(z/z_0)^2]} dz \sim \frac{\beta P_i^2 NA^2 a^2 z_0}{\pi w_0^4}. \tag{4}$$

This expression can be further simplified by using Eq. (1a)–(1g) and the definition of the critical angle, $\theta_0 = \sin^{-1}(NA) \sim NA$ for small NA. So the final expression for the returned power is

$$P_r \sim \frac{\beta P_i^2 k/\theta_0^2}{(0.65V^{1/2} + 1.619V^{-1/2} + 2.87V^{-11/2})^4}, \tag{5}$$

where k is the vacuum wave vector of the fluorescence, and V is the familiar normalized frequency of the fiber defined in Eq. (1e).

The returned power, P_r, vs. V is shown in Fig. 3. The curve has a peak which is expected since if V is too small or too large the radius of the single mode emerging from the fiber becomes too large. This leads to a drop in the pumping efficiency and hence a decrease in the returned signal. The peak occurs at $V = 3.09$ (Although this is slightly above the cut-off for the TE_{01} mode, $V \approx 2.4$, one can still align the system to excite only one mode). This maximum returned power can be attained by a trade-off between the radius, a, and the numerical aperture, NA. However, optimization is limited by the availability of fibers; a simple rule of thumb is that the optimal fiber must be chosen from commercially available single-mode fibers such that it possesses the highest NA and the lowest core radius possible. The fiber f_5_12 chosen by us is a single-mode one, not far from the optimal value of $V_{f\_5\_12} = 5.68$ (The excitation of a single mode in this fiber was verified by imaging the fiber output with a CCD camera).

In conclusion, we have demonstrated the feasibility of using a single optical fiber to produce and detect two-photon-induced fluorescence in biological markers. Our experimental results, using off-the-shelf fibers, indicate that single-mode fibers are much better suited for this fluoroprobe than multimode fibers. We established a simple model to find the optimal parameters of a single-mode fiber. This fluoroprobe opens the door for new possibilities in nonlinear spectroscopy and nonlinear near-field scanning microscopy.

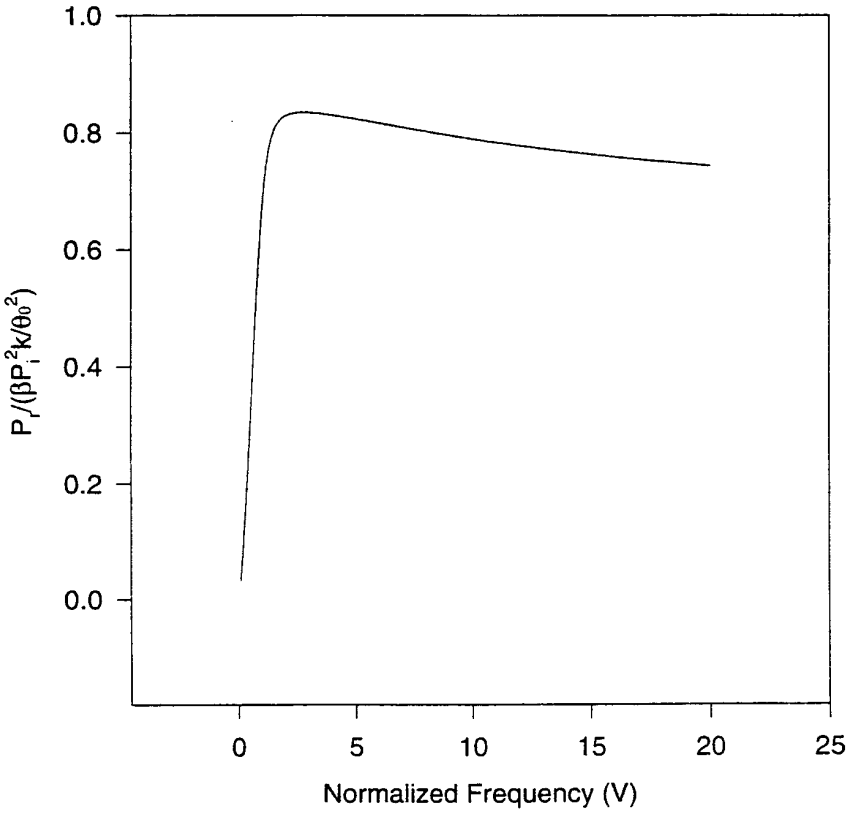

Fig. 3. Theoretical curve for the returned power, P_r (in arbitrary units), as a function of the normalized fiber frequency, V.

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