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## Optical Fiber Fluoroprobes in Clinical Analysis

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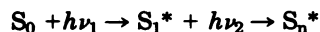
We used quartz optical fibers and laser excitation in developing single-fiber fluoroprobes, to be used to measure molecular fluorescence in minute volumes of body fluids. We illustrate and discuss the analytical capabilities of these fluoroprobes. Limits of detection for the anti-tumor drug doxorubicin are about  $10^{-7}$  mol/L, by either conventional fluorescence or sequentially excited fluorescence modes of detection. We also report the results of preliminary *in vivo* measurements of doxorubicin in the interstitial fluids of human tumors, heterotransplanted in immune-deficient laboratory mice.

**Additional Keyphrases:** *single-fiber fluoroprobes · pulsed lasers · doxorubicin · myelogenous leukemia · myelosarcoma tumors · cancer*

Conventional bioanalytical techniques often involve spectroscopic measurements of biologically important chemicals in extracted samples of body fluids or tissues. Unfortunately, close correlation between a chemical's biological activity and its concentration in an extracted sample is not guaranteed. Because physiological reactions are generally thought to occur in controlled environments of specific regions of the living body, better correlations might be expected for *in vivo* measurements made in the appropriate body regions, assuming that accuracy, precision, and sensitivity do not suffer.

The analytical capabilities of fluorescence spectrometry, which has been used in a variety of clinical analyses (1), have been extended by recent technological developments. Using quartz optical fibers, we have developed single-fiber "fluoroprobes" for microscale measurements of fluorescence *in vivo*, in extremely small volumes of body fluids, including the interstitial fluid of soft tissue. Excitation with a laser radiation source is essential with this technique. The highly collimated beam of a laser can be efficiently focused onto small optical fibers, and the high powers generally available with a laser can produce large fluorescence signals. With high-peak-power, pulsed lasers, added analytical selectivity can be exploited via detection by both linear and nonlinear excited fluorescence techniques (2).

In this work we measured concentrations of the anti-tumor drug doxorubicin by using the fluoroprobe and conventional fluorescence (CF) and sequentially excited fluorescence (SEF) techniques.<sup>1</sup> The SEF process involves sequential resonant excitation, which can be depicted as



where  $S_0$ ,  $S_1^*$ , and  $S_n^*$  are the ground, first-excited, and higher-excited singlet states of a molecule, respectively. Fluorescence is monitored from a higher excited singlet

state, instead of from  $S_1^*$  as is done in CF. The conventional and sequential excitation processes tend to populate excited states with different symmetries (3). As a result, CF and SEF can each provide unique spectroscopic information. The ability to tune both excitation and emission wavelengths is an often-stated advantage in selectivity of CF. SEF provides additional selectivity by involving three resonant transitions.

The working expression for the SEF technique, when excitation is with a continuous-wave laser, is given by:

$$I = K(P^2/A)\Phi\delta bC \quad (1)$$

where  $I$  is the signal intensity,  $K$  is a instrumental efficiency term,  $P$  is laser power,  $A$  is the cross-sectional area of the laser beam in the sample,  $\Phi$  is a fluorescence quantum efficiency term,  $\delta$  is a nonlinear absorption coefficient,  $b$  is pathlength, and  $C$  is concentration. Equation 1 can be modified for excitation with a pulsed laser, provided the pulse width of the laser is longer than the lifetime of the first excited state of the molecule involved, by simply

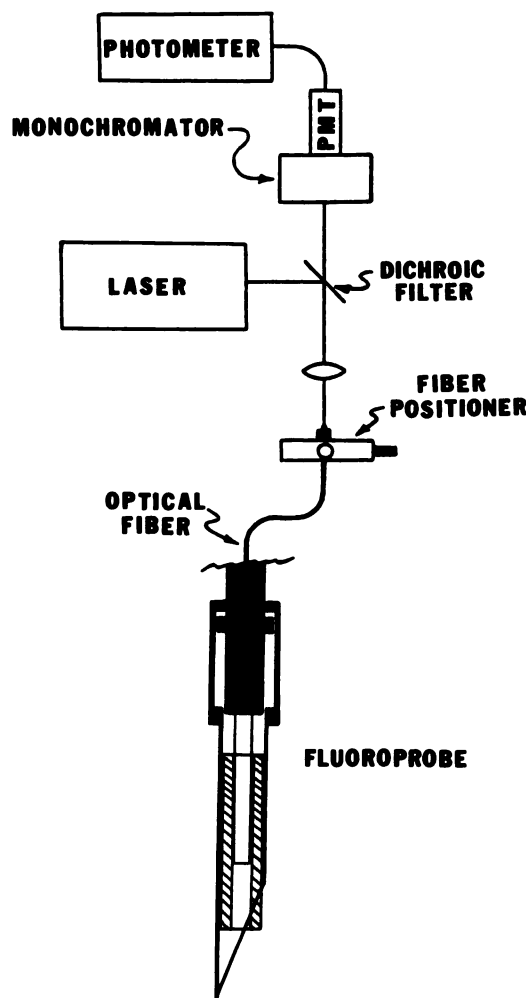


Fig. 1. Apparatus used for fluoroprobe measurements

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<sup>1</sup> Nonstandard abbreviations: CF, conventional fluorescence; SEF, sequentially excited fluorescence; HPLC, "high-performance" liquid chromatography.

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multiplying the right side of the equation by the duty cycle of the laser. When measurements are made in relatively clear matrices, by conventional techniques, the inefficiency of the SEF emission process results in sensitivities that are far inferior to CF. However, in this fluoroprobe work, we demonstrate that experimental constraints imposed by optically dense biological matrices and large amounts of stray radiation can result in comparable detectabilities for CF and SEF.

## Materials and Methods

### Apparatus

Figure 1 shows a schematic diagram of the apparatus. The laser source was either an argon ion laser (Spectra Physics, Mountain View, CA 94042; Model 171) operated at 488 nm or a nitrogen-pumped dye laser (National Research Group, Madison, WI 53705; Model NRG-0.5-5-150/B nitrogen laser and Model NRG-DL 0.03 dye laser) tuned to 480 nm by using Coumarin 481 dye. SEF measurements were made exclusively with the nitrogen-pumped dye laser, operated at 60 Hz and with average and peak powers of 8 mW and 25 kW, respectively.

The laser radiation was reflected with a dichroic filter and focused with a 25 mm, *f*/1 lens onto a quartz optical fiber. We used 200- $\mu$ m-diameter fibers (Math Associates, Port Washington, NY 11050; Model QSF-200) in this work. The optical fiber served to guide the excitation radiation into the sample and collect the fluorescence emission. An optical fiber positioner was used to situate the fiber's incident end accurately in the focused laser beam.

We used a Model CR-500-25D dichroic filter (Corion Corp., Holliston, MA 01746) for the CF measurements. This filter transmitted approximately 90% of the red-shifted CF emission. A Corion OG570 sharp cutoff filter and Model H-20 monochromator (Instruments SA, Metuchen, NJ 08840), with a 6-nm bandpass, was used to isolate the CF emission. To isolate the blue-shifted SEF emission, we replaced the sharp-cut filter and monochromator with three Model 7-54 bandpass filters (Corning Corp., Corning, NY 14830) and a 2-cm cell containing saturated  $\text{CuSO}_4$  solution. This filter combination has a bandpass of approximately 50 nm, centered at 360 nm. A Corion CR-520-25D dichroic filter, which transmitted approximately 70% of the SEF emission, was used. All fluorescence signals were detected with an RCA 1P28 photomultiplier tube operated at 800 V and processed with a quantum photometer (Pacific Precision Instruments, Concord, CA 94518; Model 126). The photomultiplier tube was cooled in solid  $\text{CO}_2$  during the SEF measurements.

We have evaluated two fluoroprobe designs for *in vivo* measurements in body fluids, differing primarily in the manner in which the body fluid is transported into the probe (see below). Two design characteristics were common to the fluoroprobes. First, their internal volumes were kept extremely small so we could perform our fluorescence measurements in the interstitial fluid of soft tissue. [It has been previously reported that interstitial fluids cannot be sampled in amounts sufficient for analysis (4).] Second, we designed the fluoroprobes to contain a well-defined optically sampled chamber (microcuvette), so that the optically sampled volume in our measurements would be constant and unaffected by changes in the biological matrix.

Samples are transported into the fluoroprobes via either aspiration or capillary action. In our early *in vivo* experiments, using a previously described fluoroprobe (5), we used elaborate sample aspiration and expulsion procedures. However, with that aspiration-type fluoroprobe it was not possible to assure that after aspiration a uniform volume of

interstitial fluid surrounded the fiber terminus, or that the fluid was completely expelled after the measurement. However, reliable sampling of interstitial fluid is possible with the fluoroprobe design shown in Figure 1. We constructed this probe, which relies primarily on capillary action to sample fluids, by first stripping the protective coating and optical cladding to a distance of 2.0 cm from the terminus of a 200- $\mu$ m diameter optical fiber. We then slid 2.0 cm of 600  $\mu$ m (o.d.)  $\times$  240  $\mu$ m (i.d.) glass capillary tubing over the stripped optical fiber and attached this assembly, with epoxy glue, into a modified 20-gauge needle. A piece of heat-shrinkable tubing was placed on the protected fiber such that the maximum distance the fiber could be withdrawn in the capillary tube was 5 mm. The available volume in the capillary tube was approximately 200 nL when the fiber was withdrawn this distance.

### Reagents

Doxorubicin hydrochloride was supplied by Adria Laboratories, Inc., Columbus, OH 43200. Blood determinations were performed with fresh whole blood, drawn in anticoagulant consisting of sodium citrate, sodium biphosphate, and dextrose, and supplied by Plasma Alliance, Knoxville, TN 37916. All doxorubicin and blood samples were protected from room light and stored at 5  $^\circ\text{C}$ .

### Procedure

Athymic nude mice with a BALB/c background were supplied by Bismarck Lozzio and James Mitchell of the Experimental Hematology and Oncology Laboratory, University of Tennessee Center for Health Science, Knoxville, TN 37996. The characteristics of these unique mutant strains of mice have been previously described (6).

Six-week-old mice of both sexes weighing 15–20 g received subcutaneous transplantations of the K-562 human myelogenous leukemia cell line developed by Lozzio and Machado (7). After seven to 14 days, myelosarcomas grew to well-vascularized masses with volumes of 1–3 mL.

The tumors of anesthetized [with Metofane (methoxyflurane)] mice were implanted with a fluoroprobe. With the capillary-action-type fluoroprobe, this was done with the optical fiber flush with the end of the glass capillary tube. For repetitive sampling and measurement we withdrew the optical fiber to allow fluid into the fluoroprobe, recorded the fluorescence signal from that sampled fluid, and then pushed the fiber back to its flush position to expel the fluid. With the aspiration-type fluoroprobe we used a 10-mL syringe, connected to the probe with Teflon tubing, to aspirate and expel the interstitial fluid.

After the background stabilized, we injected the mice intravenously with 100–200  $\mu\text{L}$  of a 3.7 mmol/L doxorubicin solution. Intravenous injection was not easy in these experiments and in certain instances direct injection into the tumor was necessary.

Fluoroprobe measurements were made over a period of several minutes. The presence of doxorubicin after successful aspiration-type fluoroprobe measurements was confirmed by extracting weighed portions of the excised tumor tissue by the procedure of Bachur et al. (8) and determining the doxorubicin in the extracts by "high-performance" liquid chromatography (HPLC) with laser fluorometric detection (9).

The exceptional sensitivity of laser fluorometric HPLC detection also made it possible to determine directly the concentration of doxorubicin in the interstitial fluid contained in the capillary action-type fluoroprobe. We removed the fluid-filled fluoroprobe from the tumor, wiped it clean, then discharged the fluid into 25  $\mu\text{L}$  of methanol. We could

then make a single injection of this diluted doxorubicin solution onto an analytical HPLC column for quantification.

## Results and Discussion

In previous work, we demonstrated that the fluoroprobe apparatus is capable of obtaining both reproducible measurements and reproducible spectra (5). In addition, we discussed how using both conventional and nonlinear excited (e.g., SEF) modes of fluorescence detection can enhance spectral selectivity. In evaluating the ultimate utility of the fluoroprobe for *in vivo* analysis, however, the analytical parameter of sensitivity must be thoroughly considered. Fluoroprobe sensitivity is perhaps the most critical determinant for obtaining *in vivo* measurements of doxorubicin. Accordingly, the following discussion is primarily devoted to evaluating the sensitivity of the fluoroprobe technique.

Analytical calibration data for doxorubicin in water and whole blood were obtained with the aspiration-type fluoroprobe, the nitrogen-pumped dye laser being used for excitation. As Figure 2 illustrates, there is an inner filter roll-off effect at high doxorubicin concentrations. This behavior is characteristic of fluorescence spectrometry.

Shorter effective pathlengths for measurements in the optically dense blood matrix (relative to H<sub>2</sub>O) result in less intense signal and smaller calibration plot slopes. However, the shorter effective pathlength also reduces inner filter problems and increases linear dynamic range. Similarly, the linear dynamic range for SEF detection is superior to that for CF detection, consistent with theoretical predictions based on the photon-density dependence of the SEF technique (see equation 1). Because the beam diverges upon

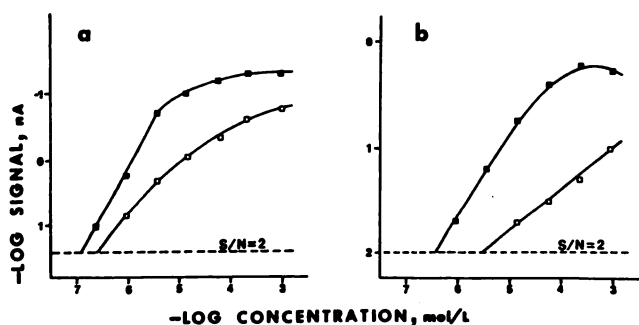


Fig. 2. Fluoroprobe-obtained calibration plots for doxorubicin by (a) CF and (b) SEF in H<sub>2</sub>O (■) and blood (□)

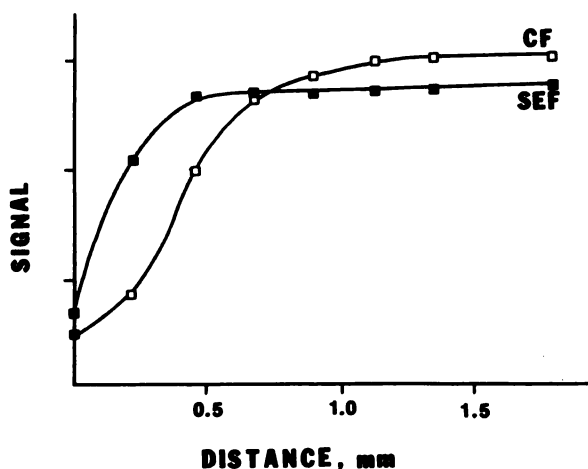


Fig. 3. Plots of SEF and CF signals (nA) for a doxorubicin solution vs optical fiber distance from a blackened surface in the solution

exiting the optical fiber, thus decreasing the photon density, the effective pathlength for SEF is shorter than for CF.

To estimate the effective pathlength for these measurements, we placed a bare, 200- $\mu$ m-diameter fiber in a beaker containing a doxorubicin solution. A layer of black, optically absorbing material covered the bottom of the beaker. Using an optical fiber positioner to translate the fiber, we recorded signals for various distances between the fiber terminus and the blackened beaker bottom (Figure 3). The effective pathlength for CF was slightly greater than 1.0 mm, and for SEF slightly less than 0.5 mm.

Limits of detection can be determined from the calibration plots in Figure 2 by extrapolating to a signal-to-noise ratio of 2. The inefficiency of the SEF emission process has resulted in far inferior detection limits for SEF, relative to CF, in liquid-chromatographic detection (3). Nevertheless, the detection limits for the fluoroprobe SEF measurements are only slightly higher than those for CF. This somewhat unexpected experimental observation can be attributed to two factors. First, the large amount of stray radiation associated with fluoroprobe measurements is efficiently rejected because of the ample blue shift between excitation and emission wavelengths in SEF detection. Similar spectral rejection is generally not observed during CF detection. During SEF detection, background levels remained near that of the dark current of the cooled photomultiplier tube, even when relatively wide-bandpass filters were used for emission collection. In CF, in contrast, the background was consistently higher and prone to greater fluctuations. Second, because of its quadratic power dependency, SEF detection better utilizes the high peak power of the nitrogen-pumped dye laser than does the CF technique, which is linearly power dependent. Using the previously described continuous-wave argon ion laser, operated at the same average power (i.e., approximately 8 mW), we could not detect doxorubicin with SEF.

The intensity of fluorescence signals generally increases when incident power is increased. However, temperature gradients, created in the sample when large amounts of absorbed radiation are dissipated as heat, can cause large increases in noise. This can be a severe problem when high-output-power lasers are used for excitation.

Using the argon ion laser, we studied the effect of increasing laser output power on signal-to-noise ratios for the CF detection of doxorubicin solutions. The optimum output power was in the range of 50 to 100 mW, higher powers producing very large background noise. The actual power in the sample was approximately 40% of the output laser power.

For most of our *in vivo* measurements, we used the CF technique and the argon ion laser, at an output power of 50 mW, for excitation. Owing to limited access to that laser, we occasionally performed *in vivo* measurements by SEF, using the nitrogen-pumped dye laser for excitation. In consideration of the quadratic power dependence of the SEF technique, we feel that SEF detection, using a pulsed laser capable of average and peak powers of approximately 100 mW and 1.0 MW, respectively (e.g., an excimer-pumped dye laser), would be the preferred method of making the *in vivo* measurements described in this report. Unfortunately, no such laser was available for this work.

The ability to use the fluoroprobe to measure doxorubicin in live tumor tissue was evaluated with the aforementioned laboratory mice. Throughout the *in vivo* work, the intravenous injection of toxic doxorubicin was a difficult experimental obstacle to overcome. Consequently, a second method of providing live tumors with a supply of doxorubicin was tried. This entailed direct injections (20–30  $\mu$ L) of the 3.7

mmol/L doxorubicin solution into the tumors. Because detection associated with intravenous injection more closely resembles clinically relevant experimental conditions, we report those results preferentially (Figure 4a, b); however, we also show fluoroprobe signals obtained via direct tumor injection (Figure 4c).

In preliminary studies, designed to determine the efficacy of the optical fiber system, we used a bare fiber implant, making no attempt to create a well-defined measurement environment at the fiber terminus. Figure 4a is the strip-chart recording of the CF signal after a 150- $\mu$ L intravenous injection of the 3.7 mmol/L doxorubicin solution. The bare 200- $\mu$ m diameter fiber was positioned approximately in the center of the tumor. A short time after injection (about 15–20 s), a relatively large, but fluctuating, increase in signal appeared. The fluctuations may have been due, in part, to animal motion (thereby changing the fiber environment) after the trauma of injection. However, the signal stabilized within a few minutes after injection. A second injection yielded similar results. Subsequent experiments were needed to determine whether the fluoroprobes would allow reproducible measurements unaffected by animal motion and capable of examining discrete volumes of interstitial fluid.

Figure 4b is a strip-chart recording of the SEF signal obtained with an aspiration-type fluoroprobe. After the 200- $\mu$ L intravenous injection, we applied suction to try to fill the

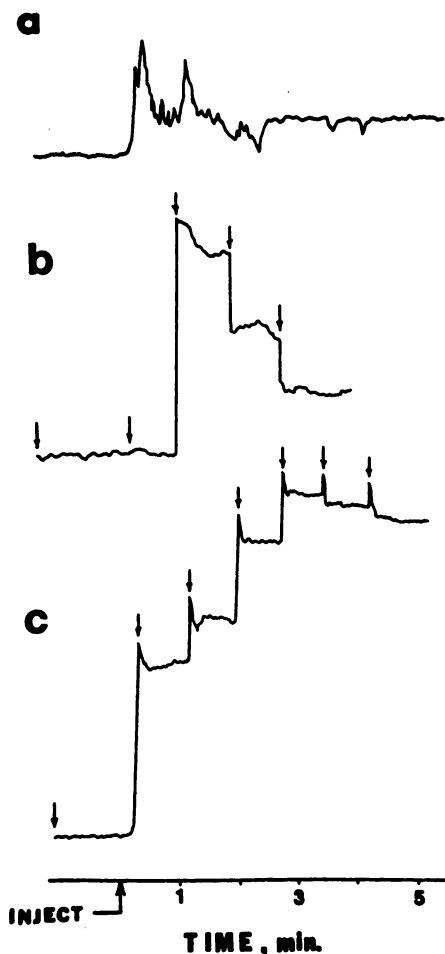


Fig. 4. Strip-chart recordings for in vivo measurements of doxorubicin in tumor by a (a) bare optical fiber, CF, intravenous injection; (b) aspiration-type fluoroprobe, SEF, intravenous injection; and (c) capillary-action-type fluoroprobe, CF, direct tumor injection. Approximate time post-injection is shown. Arrows (b and c) indicate new sample introduction into the fluoroprobe.

fluoroprobe chamber. Repetitive sampling and the signal measurement required 30–60 s, resulting in a discontinuous depiction of the increasing and decreasing concentration of doxorubicin in the tumor. Given the low background associated with SEF detection and the fluoroprobe encapsulation of the measured interstitial fluid, the fluctuations in signal observed with CF were significantly reduced. Confirmation of the presence of doxorubicin was provided by the aforementioned tissue extraction, HPLC separation, and laser fluorometric detection. Approximately 5 min after the doxorubicin injection, a section of the tumor was excised; subsequent analysis indicated the presence of 2.2  $\mu$ g of doxorubicin per gram of tumor. A tumor blank from an uninjected mouse did not show a peak on the chromatogram at the retention time for doxorubicin.

Subsequent experiments with the aspiration-type fluoroprobe revealed that while it functioned well in relatively large fluid reservoirs, it was not capable of reproducible, controlled sampling of interstitial fluids. Furthermore, in vivo signals recorded with that fluoroprobe tended at times to be noisy, presumably due to the movement of tissue fragments in the fluoroprobe during the measurement. These problems led us to design the capillary action-type fluoroprobe illustrated in Figure 1.

During in vivo analysis with the capillary action-type fluoroprobe, attempts to administer doxorubicin intravenously were unsuccessful. Direct tumor injection of approximately 30  $\mu$ L of the doxorubicin solution produced the CF signal depicted in Figure 4c. Repetitive fluoroprobe sampling indicated a rapid increase in the signal intensity, followed by a slight tapering to a relatively steady value. The injection was made about 2 mm from the fluoroprobe tip. Increases in signal were attributed to infusion of the tumor interstitial fluid near the fluoroprobe with the doxorubicin solution. Although this method of supplying the living tumor with doxorubicin may not have incorporated active transport by the animal, the capability of the capillary-action-type fluoroprobe to sample new volumes of interstitial fluid is clearly established. Previous difficulties with signal and background instability were noticeably absent, even during animal motion, when the fiber was in the withdrawn position. When we removed the fluoroprobe from the tumor and examined the probe under a microscope we could see a relatively clear fluid with no tissue fragments.

An additional advantage of the utilization of this fluoroprobe was its capability to deliver the same volume of interstitial fluid for in vitro HPLC analysis as was used to provide the in vivo fluorescence signal. As a result, we can correlate changing fluoroprobe signals with real doxorubicin concentrations, assuming a linear fluoroprobe response in dilute samples of interstitial fluid.

The actual volume of delivery of the capillary-action-type fluoroprobe used to obtain the results shown in Figure 4c was determined by discharging a fairly concentrated doxorubicin solution from the fluoroprobe into 25  $\mu$ L of methanol. We determined the concentration of the doxorubicin in this diluted solution by using the aforementioned HPLC with laser fluorometric detection. Comparison with an HPLC calibration plot for doxorubicin yielded a dilution factor of 150, from which we calculated that the effective volume of delivery of the fluoroprobe was 170 nL.

When the in vivo signal in Figure 4c had reached a steady value (approximately 5 min after injection), we removed the fluoroprobe from the tumor, examined it for tissue fragments, and discharged its contents into 25  $\mu$ L of methanol. By HPLC of this diluted sample, we calculated that its doxorubicin concentration was 2.8  $\mu$ mol/L, or 420  $\mu$ mol/L in the undiluted interstitial fluid sample. This potential for

providing independent analytical data necessary for calibrating the fluoroprobe signal, distinguishes the capillary-action-type fluoroprobe from the other design tested.

In conclusion, we have explored the potential for performing *in vivo* molecular fluorescence measurements. Through the utilization of lasers and optical fibers, we have evaluated preliminary fluoroprobe designs, and hope that the basic theoretical and experimental framework provided by this work will stimulate exploration of clinical applications for fluoroprobes.

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