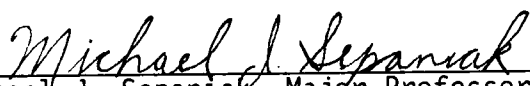
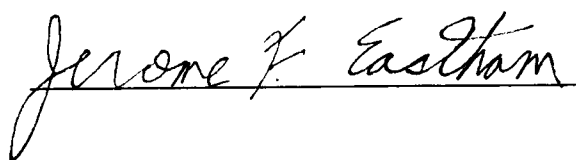


To the Graduate Council:

I am submitting herewith a thesis written by Bruce Jason Tromberg entitled "Laser-Based Optical Fiber Fluoroprobes in Clinical Analysis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.


Michael J. Sepaniak, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


The Graduate School

LASER-BASED OPTICAL FIBER FLUOROPROBES
IN CLINICAL ANALYSIS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Bruce Jason Tromberg

December 1983

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ABSTRACT

Optical fiber fluoroprobes have been designed to perform molecular fluorescence measurements in minute volumes of body fluids. The fluoroprobes are evaluated for conventional laser excited, two-photon excited, and sequentially excited modes of fluorescence detection. In-vitro investigations of test compounds in various biological matrices, including cerebral spinal fluid and whole blood, revealed comparable limits of detection (approximately 5×10^{-7} M) for the three fluorescence techniques. Linear dynamic ranges are shown to be solvent dependent and superior for the non-linear excited fluorescence techniques. The results of in-vivo measurements of the anti-tumor drug Adriamycin in tumor bearing laboratory mice are also reported. The capacity of various fluoroprobe designs to provide Adriamycin concentration measurements in tumor interstitial fluid is clearly established. In addition, independent analytical confirmation of these in-vivo measurements is provided by conventional in-vitro techniques. The ultimate detectibility and selectivity of fluoroprobe utilization is also discussed.

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INTRODUCTION

Conventional bio-analytical techniques often involve spectroscopic measurements of important biochemicals in extracted samples of body fluids or tissues. Unfortunately a close correlation between a chemical's biological activity and its concentration in extracted samples is not guaranteed. Physiological reactions are generally thought to occur in controlled environments of specific regions of the living body. Therefore, the complete effects of drugs, toxins, and naturally occurring biochemicals, as they relate to human disease and therapy, should be most accurately assessed by sensitive, in-vivo, analytical measurements made in the appropriate regions of the body.

Despite the potential analytical advantages of site-specific analyses, in-vivo measurements are bound by demanding experimental conditions which may inhibit their accuracy, precision, and sensitivity. One notable exception is the in-vivo electrochemical monitoring of neurotransmitters using microelectrodes¹.

Fluorescence spectrometry has been utilized in a variety of clinical analyses². Recent technological developments have extended the analytical capabilities of fluorescence spectrometry in clinical analysis by allowing spatially well-defined in-vivo measurements. These developments include microscale quartz optical fibers and tunable lasers with beam properties that permit focusing onto the fibers. Relatively large, precisely aligned, optical fiber

bundles have been employed as in-vivo invisioning devices (endoscopes) for biopsy and surgery³ and, using lasers, in the photochemical treatment of cancer⁴. In addition, bifurcated fiber bundles have been used to measure tissue surface fluorescence in various organs, including the brain⁵⁻⁷. In some instances efforts were made to correct for differing matrices by simultaneously monitoring surface reflectance and normalizing⁶. Furthermore, chemical reagents which produce or enhance fluorescence have been successfully immobilized at the ends of optical fibers in remote fiber fluorescence measurements⁸.

In this work, the analytical methodologies associated with the design and utilization of single fiber "fluoroprobes" for remote in-vitro and in-vivo biochemical measurements are described. Using quartz optical fibers, these microscale fluoroprobes were developed to make fluorescence measurements in micro 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 result in large fluorescence signals. In addition, the employment of conventional fluorescence (CF), two-photon excited fluorescence (TPEF), and sequentially excited fluorescence (SEF) techniques may enhance spectral selectivity during fluoroprobe detection.

CHAPTER I

STATEMENT OF THE PROBLEM

A. PROBE DESIGN REQUIREMENTS

Various fluoroprobe designs were evaluated for in-vivo measurements in body fluids, including a fluoroprobe with the capability of filtering the sampled body fluid. Two design characteristics were common to the different fluoroprobes. First, the internal volumes of the fluoroprobes were kept extremely small so as to permit in-situ sampling of interstitial fluid in soft tissue. It has been previously stated that this type of sampling was not possible⁹. Second, the fluoroprobes were designed to contain a well-defined optically sampled chamber (microcuvette). This enabled one to perform actual concentration measurements in a variety of biological matrices.

Samples were transported into the fluoroprobes via either aspiration or capillary action. Elaborate sample aspiration and expulsion procedures were employed in early experiments. However, with the aspiration-type fluoroprobe, it was not possible to assure that, after aspiration, a uniform volume of body fluid surrounded the fiber terminus, or that the fluid was completely expelled after the measurement. Reliable sampling of intestinal fluid was accomplished with the capillary action-type fluoroprobe shown in Figure 1a. Figure 1b is an exploded view of the aspiration-type fluoroprobe.

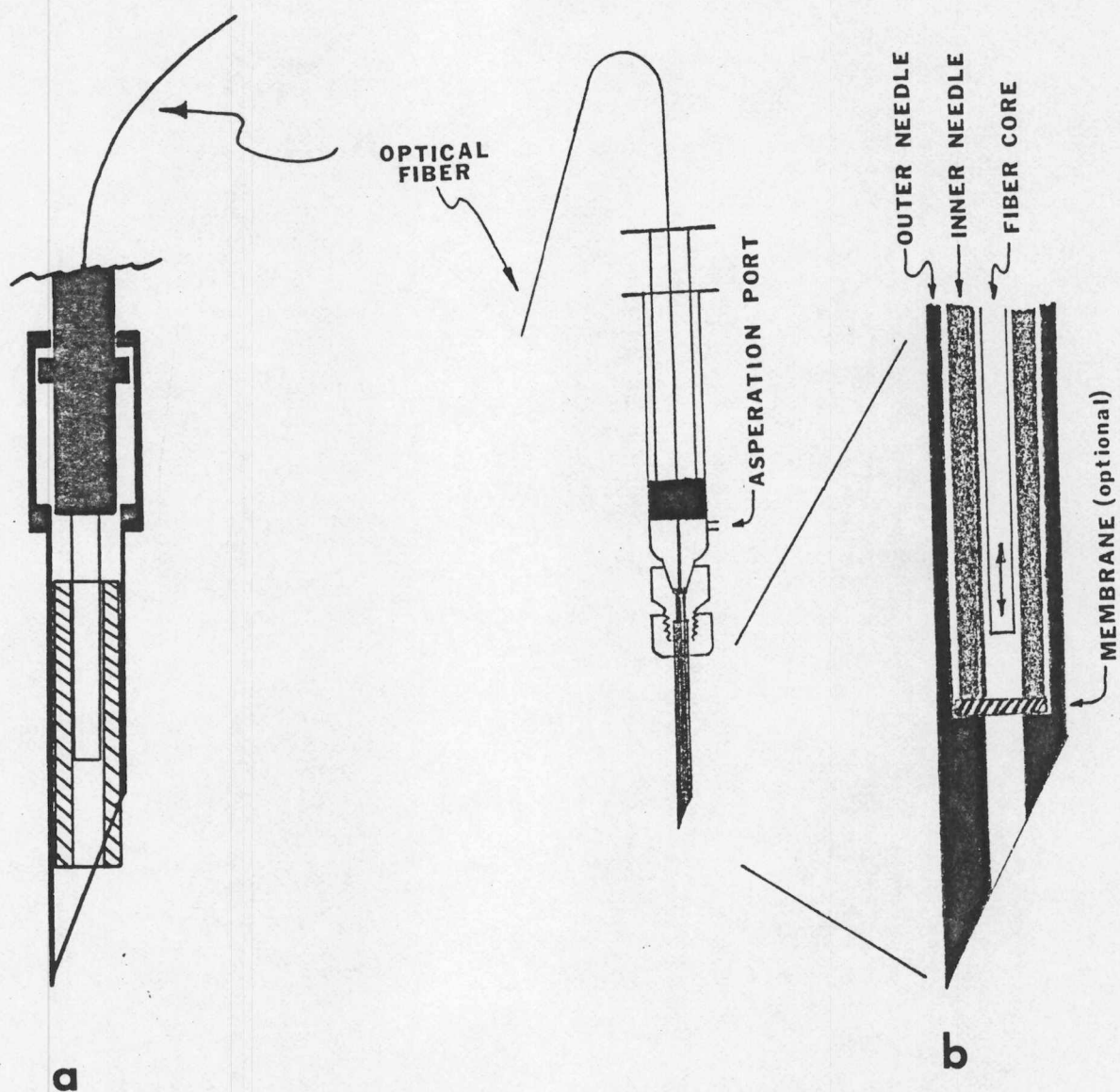


FIGURE 1. Fluoroprobe design. (a) Capillary action-type fluoroprobe. (b) Aspiration-type fluoroprobe.

Experiments with the aspiration-type fluoroprobe revealed that while it functioned well in relatively large fluid reservoirs, in-vivo signals recorded tended to be noisy, presumably due to the movement of tissue fragments in the fluoroprobe during the measurement. These problems led to the incorporation of the significantly reduced internal volume of the capillary action-type fluoroprobe.

An additional advantage to the utilization of the capillary action-type 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. This was performed by removing the probe from the animal, discharging its contents into a known volume of solvent, and injecting the sample into an HPLC apparatus. This potential for providing independent analytical data necessary for fluoroprobe signal calibration, as well as minimizing problems associated with tissue fragment interference, distinguished the capillary action-type fluoroprobe from other designs tested.

B. FLUORESCENCE DETECTION

The analytical difficulties associated with spectral interference which could not be eliminated by thoughtful probe design were minimized by the utilization of both linear and non-linear fluorescence detection.

In this work fluoroprobe measurements were made using conventional fluorescence (CF), two-photon excited fluorescence (TPEF) and sequentially excited fluorescence (SEF) techniques. The TPEF

process involves the simultaneous absorption of two photons to produce a resonant transition in a molecule¹⁰. Fluorescence is monitored from the lowest excited singlet state of the molecule (S_1^*). The SEF process involves sequential resonant excitation¹¹. This can be depicted $S_0 + h\nu_1 \longrightarrow S_1^* + h\nu_2 \longrightarrow S_n^*$, where S_0 , S_1^* , and S_n^* are the ground, first excited, and higher excited singlet states of the molecule, respectively. Fluorescence is monitored from a highly excited state. The principal analytical advantages of these two-photon excitation processes is that they are governed by different selection rules than conventional (one-photon) excitation processes¹² and this provides an added dimension for spectral selectivity. The conventional and two-photon excitation processes tend to probe excited states with different symmetries¹³. As a result, CF, SEF, and TPEF 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 due to the fact that three resonant transitions are involved.

The TPEF and SEF techniques involve relatively inefficient optical processes. Nevertheless, the low optical background of the techniques, a result of the large blue shift between excitation and emission wavelengths, and the high peak powers available with pulsed lasers, can result in reasonably sensitive detection. The working expressions for the TPEF and SEF techniques when excitation is with a continuous wave laser is given as

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

where I is the signal intensity, K is an instrumental efficiency term, P is laser power, A is the cross-sectional area of the laser beam in the sample, Φ is a fluorescence quantum efficiency term, δ is a non-linear absorption coefficient, b is path length, and C is concentration. This equation can be modified for TPEF for excitation with a pulsed laser by simply multiplying by the duty cycle of the laser. The same is true for SEF, provided that the pulsewidth of the laser is longer than the lifetime of the first excited state of the molecule involved¹³.

In normal solution measurements, the inefficiency of the two-photon emission process results in sensitivities which are inferior to CF. However, in this work, it is demonstrated that experimental constraints imposed by optically dense biological matrices, and stray radiation with the fluoroprobe, can result in comparable detectabilities for CF, SEF, and TPEF.

CHAPTER II

FLUOROPROBE IN-VITRO STUDIES

A. EXPERIMENTAL

Apparatus

A schematic diagram of the apparatus is shown in Figure 2. The laser source was either an argon ion laser (Spectra Physics Model 171) operated at 488 nm or a nitrogen-pumped dye laser (National Research Group, Model NRG-0.5-5-150/B nitrogen laser and Model NRG-DL 0.03 dye laser) tuned to 480 nm using Coumarin 481 dye. SEF measurements were made exclusively with the nitrogen-pumped dye laser, which was operated at 60Hz and had average and peak powers of 8 mW and 25kW, respectively.

A 25-mm focal length, f/1 lens was used to focus the laser radiation onto the end of a 200 μ m core diameter, quartz, optical fiber (Math Associates Inc. Model QSF-200). A single fiber was employed to guide the excitation radiation into the sample, collect the fluorescence emission (the fiber has a total acceptance angle of 25°), and guide the emission towards the PMT. An optical fiber positioner was used to accurately situate the fiber's incident end in the focused laser beam. The fiber terminus was enclosed in the fluoroprobe.

For the CF measurements, the dichroic filter employed was a Corion CR-500-25D. This filter transmitted approximately 90% of

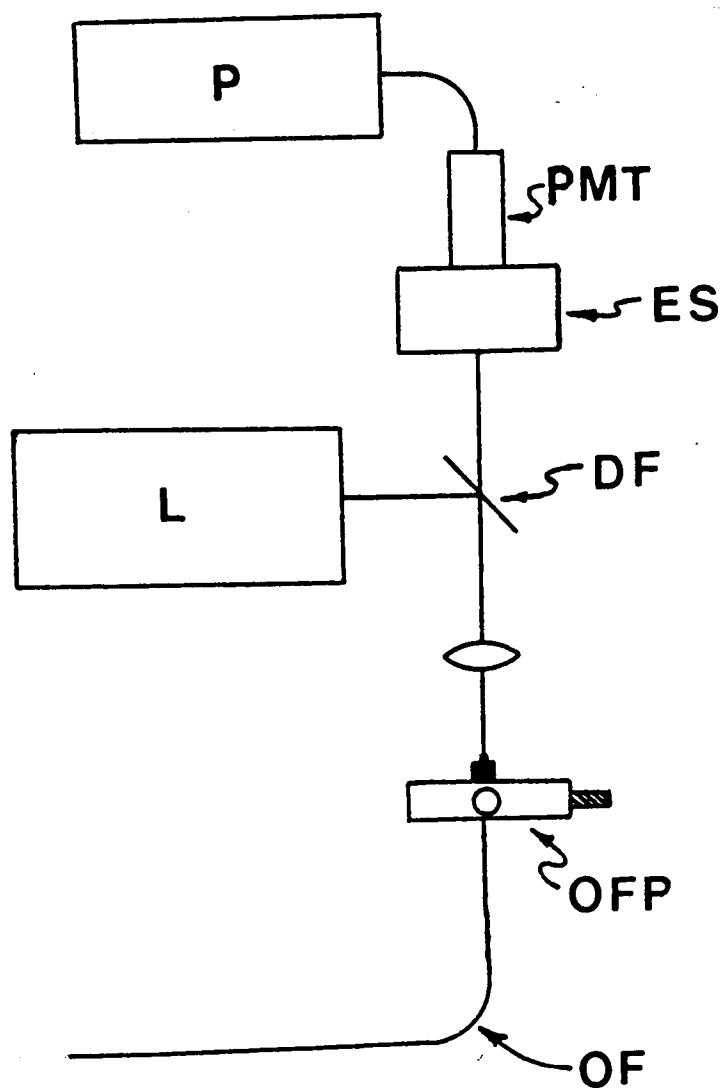


FIGURE 2. Apparatus: P, quantum photometer; PMT, photomultiplier tube; ES, emission selector (monochromator or optical filters); DF, dichroic filter; OFP, optical fiber positioner, OF, optical fiber.

the red-shifted CF emission. A Corion OG570 sharp cut-off filter and an Instruments SA Model H-20 monochromator, with a 6-nm band-pass, was used to isolate the CF emission. In order to isolate the blue-shifted SEF emission the sharp cut-off filter and monochromator were replaced by three Corning 7-54 bandpass filters and a two-cm cell containing a saturated 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 volts and processed with a Pacific Precision Instruments Model 126 quantum photometer. The photomultiplier tube was dry ice cooled during the SEF measurements. In addition, a strip chart recorder was employed to accurately determine noise levels.

Fluoroprobe

A diagram of the aspiration-type fluoroprobe, including an exploded view of the syringe needle tip, is shown in Figure 1b, page 4. The fully clad fiber was threaded through a 1.0 cc plastic tuberculin syringe. The fiber's outer coatings were stripped at the fibers sampling end and the bare 200- μm core was cut and placed in a blunt-end, 26-gauge needle. The 26-gauge needle was then slipped into a bevel-end, 21-gauge needle that had been modified to hold an optional micropore filter. A filter was not employed in the work reported herein.

A side port linked the fiber-containing syringe to a 3.0-mL plastic syringe. The processes of fluid aspiration, expulsion, and termination of flow, were accomplished by the combined movements of each syringe plunger. Adjustments in the position of the tuberculin plunger determined the location of the fiber's sampling terminus. When the fiber was withdrawn a known distance, a sampling chamber (i.e., cuvette) was created within the needle. As a result, calibrated, preset fluoroprobes provided constant reproducible volumes of analyte. With the sampling terminus positioned 3.0 mm above the needle level, only 0.4 μ L of fluid was needed to uniformly fill the sampling chamber.

Materials

All blood determinations were performed with fresh whole blood, drawn in the anticoagulant C.P.D. (sodium citrate, sodium biphosphate, and dextrose), and supplied by Plasma Alliance, Knoxville, Tennessee. Cerebral spinal fluid (CSF) samples were kindly provided by Dr. P. Painter of the University of Tennessee Medical Center. The non-biological solvents were HPLC grade methanol (Fisher Chemical Co.) and distilled water. The fluorescent analytes used were the antitumor drug doxorubicin hydrochloride (Adriamycin) and 2-(4-biphenyl)-5-phenyl-1,3,4-oxadiazole (PBD). Structures are shown in Figure 3. Adriamycin (ADR) was supplied by Adria Laboratories Inc., Columbus, Ohio. PBD was obtained through Pfaltz and Bauer Inc., Stamford, Connecticut.

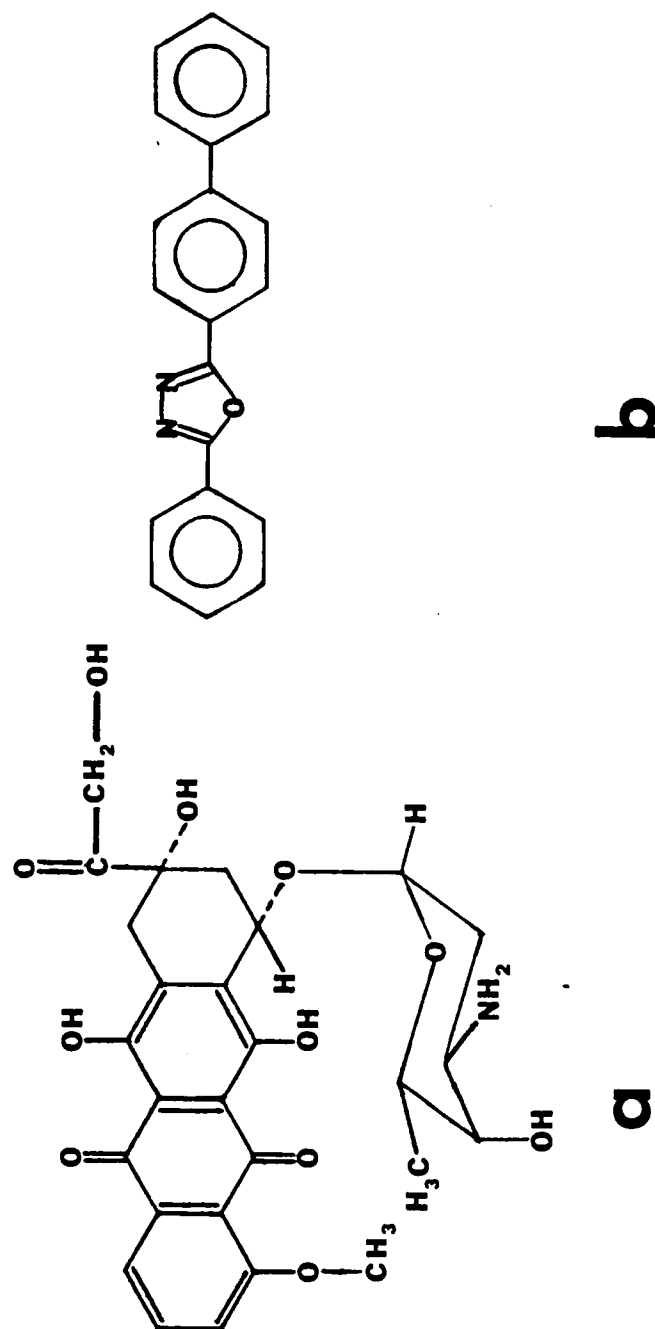


FIGURE 3. Structure for: (a) doxorubicin (ADR), (b) 2-(4-(4-biphenyl)-5-phenyl-1,3,4-oxadiazole (PBD)).

In order to minimize photolytic and thermal degradation¹⁴, all ADR and biological fluid samples were protected from light and refrigerated at 5°C. Each solvent was pooled, and serial dilutions of the analytes were made 24 hours prior to measurement.

B. RESULTS AND DISCUSSION

Unique applications of advanced analytical spectroscopy can provide detection advantages in demanding biological measurements. As a result, the ultimate analytical value of fluoroprobe detection is best determined by a thorough examination of its suitability to both linear and non-linear spectroscopic phenomena. The following discussion will therefore address the general analytical utility of fluoroprobe design, as well as its applicability to various fluorescence detection techniques. In addition, calibration plots for fluoroprobe measurements involving CF, SEF, and TPEF detection will be evaluated for sensitivity, linearity of response, and the impact of matrix optical density. The potential spectroscopic selectivity of the detection techniques will also be discussed.

Spectra

A point by point emission scan was obtained via fluoroprobe sampling of ADR/H₂O and ADR/CSF solutions. A Corion OG-570 sharp cut-off filter was used to minimize optical background. Figure 4 reveals obvious similarities between the fluoroprobe spectra and a conventional scan obtained using an Aminco-Bowman spectrofluorimeter with a Corion OG-570 filter. Each emission spectrum is roughly

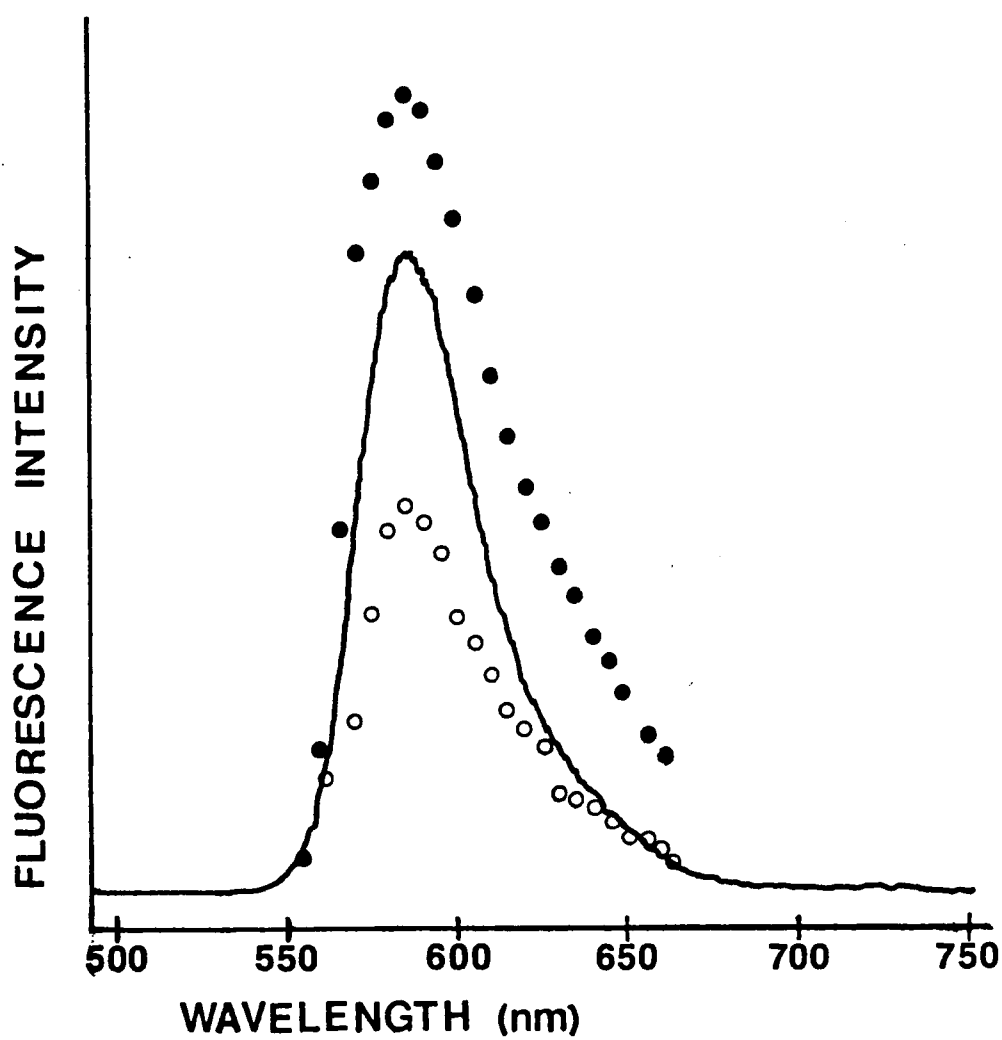


FIGURE 4. Fluorescence intensity versus wavelength (nm) for: ADR/H₂O, Aminco Bowman; • ADR/H₂O, Fluoroprobe; o ADR/CSF, Fluoroprobe.

centered at 585 nm and is consistent with previously reported spectra¹⁵. This demonstrates the reliability of the fluoroprobe for obtaining spectra in regions for which the optical fiber is transparent.

Reproducibility

Replicate CF fluoroprobe determinations were performed on 2×10^{-4} M solutions of ADR in water, CSF, and blood. The relative standard deviations for four determinations in each of the solvents were 0.05%, 1.68%, and 1.19%, respectively. These data suggest good fluoroprobe measurement reproducibility in a variety of matrices.

Calibration Plots

Linearity. The CF curves in Figure 5a exhibit early inner filter roll-off behavior at high ADR concentrations. That Figures 5b and 5c reflect a greater linear dynamic range is consistent with theoretical predictions. At high fluorophor concentrations, solute photon density destroys the linearity of conventional fluorescence calibration. This photon density effect is somewhat mitigated during non-linear processes, when $I \propto \frac{I}{A}$ (equation 1). During SEF and TPEF, the greatest non-linear signal contribution is from the area of highest photon density, or smallest beam size. Since the beam diverges upon exiting the optical fiber, the value of "A" is smallest, and "I" largest, close to the optical fiber terminus. The shortened effective path length at high solute concentrations should therefore result in a less severe linearity breakdown for SEF and TPEF than for the more path-length-dependent CF process.

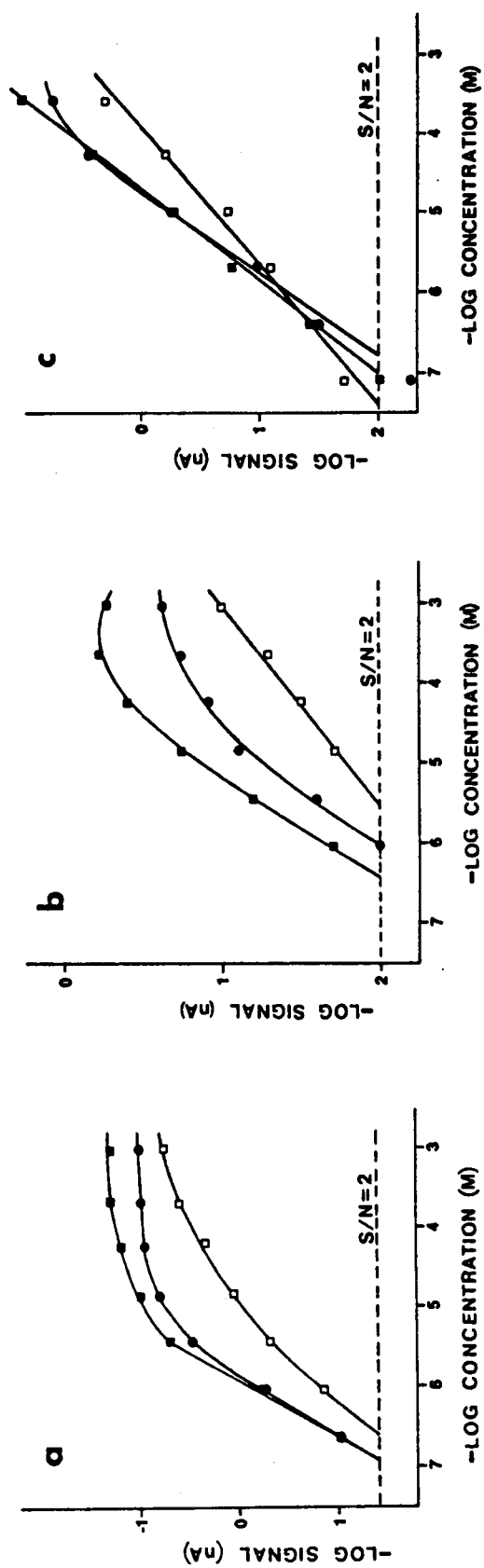


FIGURE 5. -Log signal (na) versus -log concentration (M) for: (a) CF of ADR, (b) SEF of ADR, (c) TPEF of PBD. Three matrices: $\blacksquare$ H₂O, $\bullet$ CSF, $\square$ Blood.

The superior linearity of SEF and TPEF in optically dense solvents (blood, CSF) can be similarly explained. Measurements of ADR concentrations in blood are susceptible to strong matrix reduction of effective path length. As a result, fluorescence intensity ceases to be a simple function of fluorophor concentration, and the CF blood curve displays the expected inner filter roll-off. In contrast, the high solute concentration portion of the SEF and TPEF curves remains linear, despite high medium optical density. This apparent absence of inner filter effect is in agreement with the proposed reduced path length dependency of non-linear fluorescence processes.

Slope. It is evident from Figure 5 that calibration plot slopes, and hence sensitivities for fluoroprobe measurements, vary according to solvent and detection technique. One characteristic common to both linear and non-linear fluorescence processes is a gradual decrease in calibration plot slopes as the solvent changes from H₂O to CSF to blood. This can be attributed to solvent absorption properties. Whole blood contains two absorption bands (Figure 6) which effectively bracket regions of sequential and conventional fluorescence. As a result, a constant percentage of signal is reabsorbed by solvent.

Blood's strongest absorption band is, at 415 nm, in the region of the SEF signal. Consequently, the disparity between calibration lines for H₂O and blood is greater during SEF. Similarly, the CSF pool used was found to be slightly hemolyzed. The strong

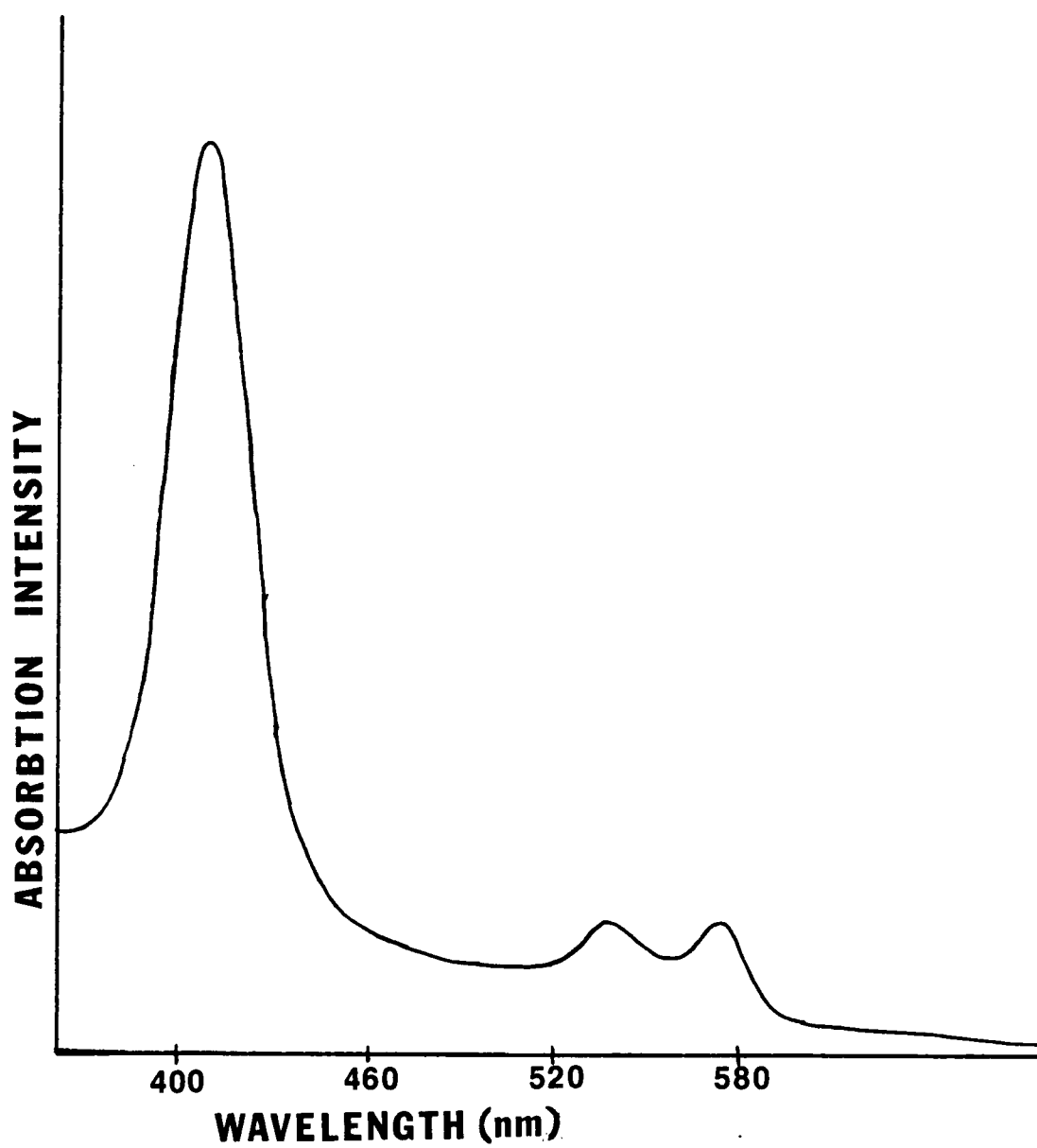


FIGURE 6. Absorbion intensity versus wavelength (nm) for Blood/
 H_2O .

signal absorption capabilities of even a few red cells causes a sizeable slope difference between the H₂O and CSF lines during SEF (Figure 5b). In addition, the weaker blood absorption band at 585 nm is responsible for the less pronounced, but constant, signal reduction of CSF during conventional fluorescence (Figure 5a).

This signal reabsorption effect does not appear during the measurements of PBD in blood and CSF presumably due to much less of an overlap between the TPEF emission spectrum of PBD, relative to the SEF emission spectrum of ADR, and the very strong 415 nm absorption band present in blood and hemolyzed CSF.

Detectability

The limits of detection (LOD) shown in Table 1 were obtained by extrapolating the calibration plots to a signal to noise ratio (S/N) of 2. The LOD values are comparable for the three fluorometric techniques despite the inherent inefficiencies of the SEF emission and TPEF excitation processes. This can be attributed to two causes. First, the quadratic power dependency of the non-linear fluorometric techniques better utilizes the relatively high peak powers of the pulsed laser employed in this work. Using the previously described, continuous wave, argon ion laser, operated at the same average power (i.e., approximately 8 mW), the CF detection of ADR was not possible.

Second, the large blue-shift between excitation and emission wavelengths for the non-linear techniques facilitates spectral rejection of specular, Raman, and Rayleigh scatter. A large amount of specular scatter is observed with the fluoroprobe, particularly when

TABLE 1. Fluoroprobe Limits of Detection

	ANALYTE	MATRIX		
		H ₂ O/CH ₃ OH	CSF	BLOOD
CF	ADR	$1.3 \times 10^{-7} \text{M}$	$1.3 \times 10^{-7} \text{M}$	$2.5 \times 10^{-7} \text{M}$
SEF	ADR	$3.6 \times 10^{-7} \text{M}$	$1.0 \times 10^{-6} \text{M}$	$2.8 \times 10^{-6} \text{M}$
TPEF	PBD	$1.0 \times 10^{-7} \text{M}$	$1.6 \times 10^{-7} \text{M}$	$4.0 \times 10^{-8} \text{M}$

measurements are made in dense matrices. During non-linear excited fluorescence detection, typical background levels were near or equal to the PMT dark current, despite our use of relatively wide band-pass emission selection filters. The utilization of a wide emission bandpass for the CF measurements was not possible due to a concomitant high background which tended to cause detector saturation. Of course, the SEF and TPEF detectabilities would suffer if emission bandpasses were narrowed in order to obtain emission spectra.

Fiber shape. Efforts were made to modify the optical fiber terminus in order to achieve a focusing effect. This should serve to improve fluorescence collection efficiency for all the fluorometric techniques, since it would increase the fraction of emission that occurs immediately in front of the optical fiber. Moreover, it should increase the total amount of SEF and TPEF emission due to the dependence of those techniques on photon density. Similarly, photon density can be increased through the use of smaller diameter optical fibers. However, at some point, reductions in fiber diameter can result in poor coupling of the laser radiations onto the fiber.

After aligning an optical fiber, its terminus was cut square. The fiber was placed in a PBD solution and the TPEF signal recorded. The terminus was shaped into a hemisphere by heating with a microtorch and the TPEF signal recorded again. Another reading was made after melting the fiber terminus into a bulb with a diameter approximately twice that of the fiber. This procedure was repeated three times. The results of this study were somewhat discouraging. The average

signals for the square-cut, hemispherical, and bulb-shaped termini were 18 nA, 23 nA, and 11 nA, respectively. A decrease in the transparency of the terminus as a result of the torch heating and an increase in critical angle internal reflection at the hemispherical and bulb-shaped termini may, at least partially, account for the discouraging results.

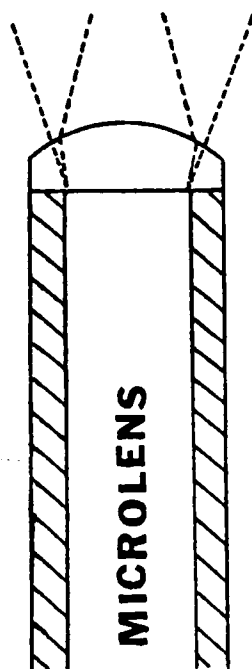
A miniaturized version of the sapphire ball optrodes employed by Hirschfeld⁸, or a microlens¹⁶ coupled to the end of the fiber, could have proved more successful. As a result, a second study was performed in order to assess the influence on SEF signal levels of a 2.0-mm diameter, 1.6-mm focal length microlens afixed to the fiber terminus. In addition, the effects of varying optical fiber diameters were examined. The relative signal levels shown in Figure 7 were obtained using 0.2-mm, 0.6-mm, and 1.0-mm core diameter fibers. The results indicate that 0.2-mm, or smaller, diameter fibers, coupled with a suitably sized microlens, may prove to be the most efficient means of making fluoroprobe measurements, particularly those based on non-linear excited fluorescence. Unfortunately, these lenses are not currently commercially available.

Effective path length. Shorter effective path lengths for measurements in the optically dense blood matrix (relative to H₂O) result in lower signal levels and smaller calibration plot slopes. However, the shorter effective path length reduces inner filter problems and increases linear dynamic range. Similarly, the linear dynamic range is superior for SEF detection relative to CF detection.

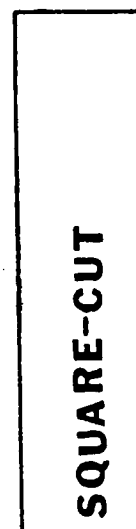
FIGURE 7. Relative signal levels for various optical fiber diameters and different fiber terminus shapes.

RELATIVE SIGNAL [nA]

0.2 mm	0.6 mm	1.0 mm
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1.1



0.7



—

This is consistent with the previously described theoretical predictions based on the photon density dependence of the SEF technique (see equation 1).

In order to estimate the effective path length for these measurements, a bare 200- μm core diameter fiber was placed in a beaker containing an ADR solution. A layer of black, optically absorbing material was affixed to the bottom of the beaker. Using an optical fiber positioner to translate the fiber, SEF and CF signals were recorded for various distances between the fiber terminus and the blackened beaker bottom. The results in Figure 8 indicate an effective path length for CF of slightly greater than 1.0 mm and for SEF of slightly less than 0.5 mm.

Laser power. Further improvements in detectability can be expected using lasers with higher output powers. Thermal problems, however, may ultimately limit power related improvements in detectability. The results of a study on the influence of laser power on detectability are presented in Figure 9. In this work, the argon ion laser was focused onto a bare 200- μm core diameter optical fiber.

Strip chart recordings of CF solvent background levels and ADR solution signal levels are shown for two different solvents and at three incident laser powers. The actual power in the sample was approximately 40% of the incident laser power. An ADR in H_2O solution yielded a large increase in S/N when the incident laser power was increased from 5 mW to 50 mW, but a much smaller increase in S/N

FIGURE 8. Plots of (b) SEF and (a) CF signal levels of an Adrimycin solution versus optical fiber distance from a blackened surface in the solution.

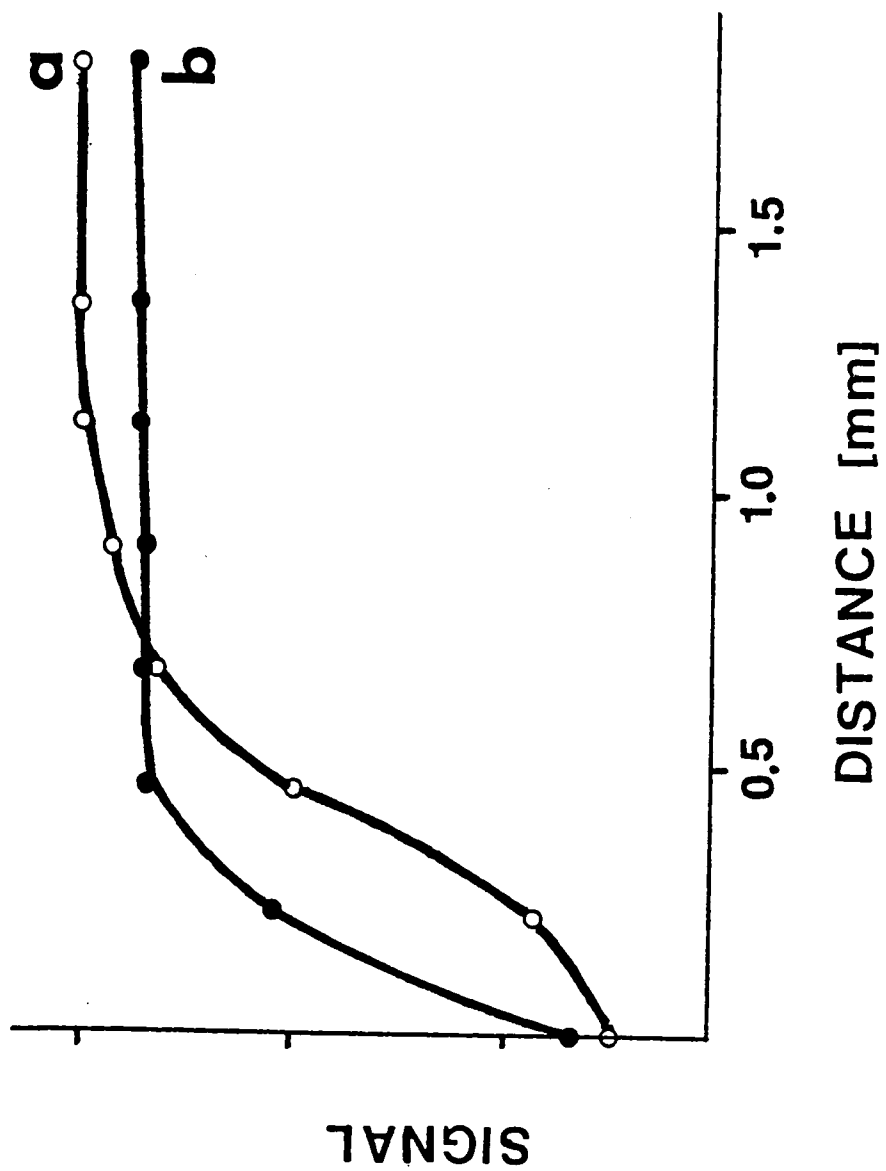
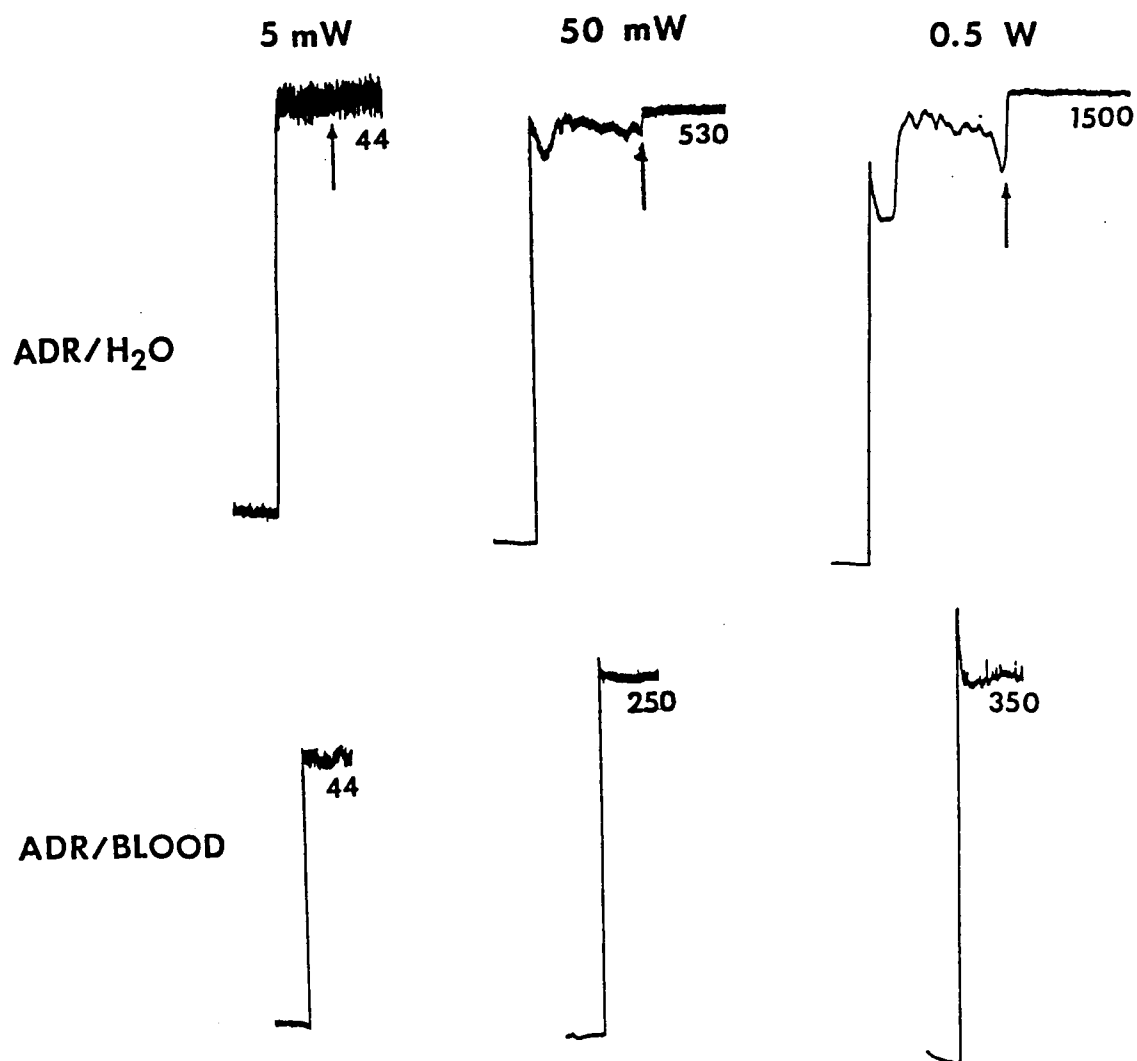


FIGURE 9. Strip chart recordings demonstrating the influence of laser incident power on S/N (numbers shown on tracings) for H₂O and blood solvents. The arrows indicate the introduction of stirring in the H₂O solution.



was observed when the power was elevated from 50 mW to 0.5 W. An ADR in blood solution showed considerably smaller increases in S/N with increasing power. These effects can presumably be attributed to various thermal problems, including thermal diffusion of the ADR from the optical fiber terminus.

In order to investigate the thermal diffusion problem, the ADR in H₂O solution was stirred during a portion of the measurement. Fluorescence signals stabilized significantly at higher laser powers when this was done. As a result, it was concluded that the appearance of signal drift in the unstirred solutions can be attributed to slight movements of the optical fiber terminus within a thermal diffusion produced ADR concentration gradient.

These thermal effects may be minimized by employing SEF and TPEF detection. Sample heating depends on the average laser power, which, for a pulsed laser, is roughly equal to the product of the peak power and the laser duty cycle. Signal levels for SEF and TPEF are proportional to the product of the peak power squared and the laser duty cycle. Consequently, laser changes which provide increases in peak power and decreases in duty cycle can raise signal levels for the non-linear techniques without increasing average laser power.

Selectivity

The trace level fluorometric determination of important biochemicals in body fluids will place challenging requirements on the selectivity of the spectroscopic techniques involved. The fact that

ADR has excitation and emission wavelengths in the visible greatly simplified its measurement in this work. Nevertheless, broad absorption bands in the biological matrices influenced the shape of the ADR calibration plots. When the pertinent spectroscopic bands of the biochemical of interest are at wavelengths less than about 300 nm, certain fluorescent components in biological matrices (e.g., tryptophan) will seriously interfere with the measurement. In those, and less demanding situations, the simultaneous utilization of CF, SEF, and TPEF modes of detection may provide substantial advantages in selectivity, facilitating spectral discrimination between biochemicals in body fluids. The comparable fluoroprobe sensitivities of these modes of detection has been demonstrated in this work.

There are several possibilities for enhancing selectivity by combining the CF, SEF, and TPEF techniques. The conventional (one-photon) excitation process tends to populate unsymmetric excited singlet states, while the non-linear excitation processes tend to populate symmetric excited singlet states¹². Thus, the potential for spectral resolution is improved when both processes can be utilized. The often stated selectivity advantage in CF of being able to tune both excitation and emission wavelengths is taken one step further in SEF, where three resonant optical transitions can be individually tuned. In addition, the TPEF technique has the advantage that excitation into UV lying lowest excited states is done with visible radiation. Biological samples generally contain

many more UV absorbing components than visible absorbing components.

Of course, the TPEF emission can still be in the UV region. It is felt that these unique aspects of CF, SEF, and TPEF may significantly enhance the selectivity of fluoroprobe measurements.

CHAPTER III

FLUOROPROBE IN-VIVO STUDIES

A. EXPERIMENTAL

Apparatus and Reagents

The apparatus and reagents used for in-vivo analyses were identical to those employed during in-vitro studies. A complete description may be found in Chapter II-A.

Fluoroprobe

The in-vivo experiments were performed with the aspiration-type and capillary action-type fluoroprobes. The aspiration-type fluoroprobe design is described in Chapter II-A.

The capillary action-type fluoroprobe, illustrated in Figure 1a, page 4, was constructed by first stripping the protective coating and optical cladding to a distance of 2.0 cm from the terminus of a 200- μm core diameter optical fiber. A 2.0-cm length of 600- μm O.D. x 240- μm I.D. glass capillary tubing was then epoxied into a modified 20 gauge needle. A piece of heat shrinkable tubing was placed on the protected fiber so 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.

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, Tennessee. The characteristics of these unique mutant strains of mice have been previously described¹⁷.

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 Machado¹⁸. After 7-14 days, myelosarcomas grew to well vascularized masses with volumes of 1-3 cm³.

The tumors of Metophane-anesthetized mice were implanted with a fluoroprobe. When using the capillary action-type fluoroprobe, this was done with the optical fiber flush with the end of the glass capillary tube. Repetitive sampling and measurement was accomplished by withdrawing the optical fiber, recording the fluorescence signal, and then pushing the fiber back to its flush position. With the aspiration-type fluoroprobe a 10-cc syringe, connected to the probe with teflon tubing, was used to aspirate and expell the interstitial fluid.

In order to maximize the detectability in a given fluoroprobe measurement, it was necessary to select an appropriate laser power. Consideration of the in-vitro laser power study revealed that the determination of optimum laser power is critically dependent on the exact biological matrix being investigated. As a result, capillary

action-type fluoroprobe in-vivo measurements of ADR were performed using CF detection, while employing the argon ion laser operated at 50 mW. A pulsed laser with similar average power (e.g., an excimer-pumped dye laser) could possibly have yielded better results using SEF detection. Unfortunately, such a laser was not available for this work. All aspiration-type fluoroprobe in-vivo measurements were performed using SEF detection with the Nitrogen-pumped dye laser operated at 8 mW.

After observing a stable background level, the mice were injected intravenously with 100-200 μ L of a 2.0 mg/mL ADR solution. Intravenous injection was not easily accomplished in these experiments and, in certain instances, direct tumor injection was necessary.

Fluoroprobe measurements were made over a period of several minutes. The presence of ADR after successful aspiration-type fluoroprobe measurements, was confirmed by extracting weighed portions of the excised tumor tissue, using the procedure of Bachur and co-workers¹⁵. The ADR in the extracts was subsequently determined via HPLC with laser fluorometric detection¹⁹.

The exceptional sensitivity of laser fluorometric HPLC detection also made it possible to directly determine the concentration of ADR in the interstitial fluid contained in the capillary action-type fluoroprobe. This was accomplished by removing the fluid-filled fluoroprobe from the tumor, wiping it clean, then discharging the fluid into 25 μ L of methanol. A single injection of this diluted ADR solution, into an analytical HPLC column, was then possible.

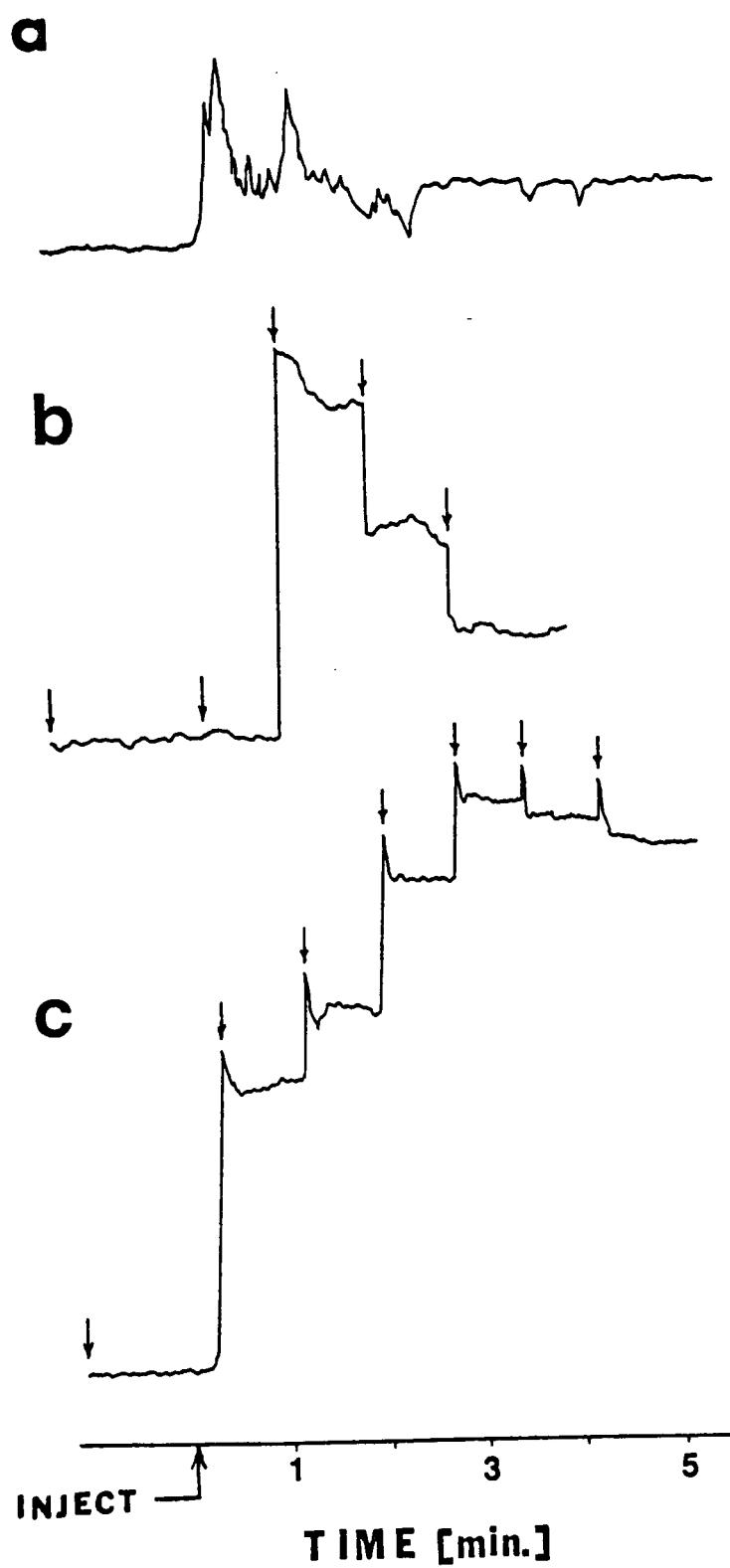
B. RESULTS AND DISCUSSION

The results of the various in-vitro studies clearly demonstrated the capability of the fluoroprobe to detect ADR in certain biologically significant matrices. comprehensive in-vivo experimentation was necessary, however, in order to evaluate the fluoroprobe's ultimate clinical utility. This was accomplished by performing ADR measurements in the interstitial fluids of live tumor tissue using the aforementioned laboratory mice, and employing different fluoroprobe designs.

Throughout the in-vivo work, intravenous injection of ADR proved to be a difficult experimental obstacle to overcome. Consequently, a second method of providing live tumors with a supply of ADR was used. This entailed direct injection (20-30 μL) of the 2.0 mg/mL ADR solution into the tumors. Because detection associated with intravenous injection of ADR more closely resembles clinically relevant experimental conditions, these results are preferentially reported (Figures 10a and 10b). Due to the previously mentioned difficulties associated with intravenous injection, however, Figure 10c shows fluoroprobe signals obtained via direct tumor injection.

Preliminary studies designed to determine the efficacy of the optical fiber systems were performed using a bare fiber implant. No attempt was made to create a well-defined measurement environment at the fiber terminus. Figure 10a is the strip chart recording of the CF signal following a 150 μL intravenous injection of the

FIGURE 10. Strip chart recordings for in-vivo tumor measurements of ADR for (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 post-injection time scale is shown. Arrows for b and c indicate new sample introduction.



2.0 mg/mL ADR solution. The bare 200 μm core diameter fiber was positioned approximately in the center of the tumor. A short time after injection (approximately 15-20 seconds), a relatively large, but fluctuating, increase in signal appeared. This may have been due, in part, to animal motion (thereby changing the fiber environment) following the trauma of injection. However, the elevated signal stabilized within a few minutes after injection. A second injection yielded similar results. Although a blank injection was not performed in this study, it was concluded that optical fibers could be used for the in-vivo detection of ADR actively transported into tumor interstitial fluid. Subsequent experiments were needed, however, which would allow reproducible measurements unaffected by animal motion and capable of examining discrete volumes of interstitial fluid.

Figure 10b is a strip chart recording of the SEF signal obtained using an aspiration-type fluoroprobe. Following a 200- μL intravenous injection, suction was applied in an attempt to fill the fluoroprobe chamber. Repetitive sampling and signal measurement required 30-60 seconds. For this reason, a discontinuous depiction of the increasing and decreasing concentration of ADR in the tumor was observed. It should be noted, however, that a similar rapid appearance of ADR in the tumor was observed for the bare fiber and this fluoroprobe.

Due to the low background levels with SEF detection and the fluoroprobe encapsulation of the measured interstitial fluid, the previously observed fluctuations in signal were significantly reduced

Unfortunately, this fluoroprobe was incapable of removing consistent micro-volumes of fluid for further analysis. Consequently, independent confirmation of the presence of ADR was provided by the aforementioned tissue extraction, HPLC separation, and laser fluorometric detection techniques. Approximately 5 minutes after injection a section of the tumor was excised. Subsequent analysis indicated the presence of 2.2 ppm ADR in the tumor. A tumor blank from another mouse did not show a peak at the ADR retention time.

During in-vivo analysis with the capillary action-type fluoroprobe, attempts to administer ADR intravenously were unsuccessful. Direct tumor injection of approximately 30 μ L of the 2.0 mg/mL ADR solution produced the CF signal depicted in Figure 10c. Repetitive fluoroprobe sampling resulted in a rapid rise in the signal level followed by a slight tapering off to a relatively steady value. Since the injection was made roughly 2 mm from the fluoroprobe tip, signal elevations were attributed to the infusion of the tumor interstitial fluid near the fluoroprobe with ADR. Though this method of supplying the living tumor with ADR 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 level instability were noticeably absent, even during animal motion, when the fiber was in the withdrawn position. Examination of the fluoroprobe under a microscope revealed the presence of a relatively clear fluid with no tissue fragments.

The actual volume of delivery of the capillary action-type fluoroprobe used to obtain the results shown in Figure 10c was determined by discharging a fairly concentrated ADR solution from it into 25 μL of methanol. The ADR concentration of this diluted solution was then determined using the HPLC laser fluorometric detection technique. Comparison with an ADR calibration plot yielded a dilution factor of 150, or an effective volume of delivery of 170 nL.

When the in-vivo signal in Figure 10c had reached a steady value (approximately 5 minutes after injection), the fluoroprobe was removed from the tumor, examined for tissue fragments, and discharged into 25 μL of methanol. HPLC analysis of this diluted sample yielded a concentration of $2.8 \times 10^{-6}\text{M}$ ADR, or $4.2 \times 10^{-4}\text{M}$ ADR in the interstitial fluid sampled most recently by the fluoroprobe, assuming a linear fluoroprobe response. This capacity for simple and rapid correlation of changing fluoroprobe signals with real ADR concentrations, via an established analytical technique, is unique to the capillary action-type fluoroprobe.

CHAPTER IV

CONCLUSIONS

The potential for performing in-vivo molecular fluorescence measurements has been explored in an extensive series of fluoroprobe in-vitro experiments. Through these investigations, it was determined that the fluoroprobe apparatus was capable of obtaining both reproducible measurements and spectra. The utilization of conventional as well as non-linear excited modes of fluorescence detection to enhance spectral selectivity was also discussed. In addition, the analytical parameter of sensitivity, perhaps the most critical determinant of the fluoroprobe's in-vivo utility, has been thoroughly considered in a variety of biological matrices.

The basic theoretical and experimental framework provided by these encouraging in-vitro experimental results stimulated the exploration of fluoroprobe clinical applications. As a result, several in-vivo investigations were performed which clearly indicated the importance of probe design and fluorescence detection in the determination of clinical sensitivity.

By utilizing SEF detection and high peak power pulsed lasers, shorter effective path lengths, minimal sample heating effects, and low optical background levels were observed. These potential sensitivity enhancement factors proved to be particularly useful in biological measurements, where the medium's heterogeneity and optical density contributed to stray radiation and thermal problems.

With the development of a capillary action-type fluoroprobe capable of reproducible, unique, micro-volume sampling for both in-vivo and in-vitro analyses, the potential for additional clinical applications increased greatly. It is hoped that the sensitivity and selectivity advantages provided by the novel combination of laser induced fluorescence detection with a highly maneuverable, low volume, sampling fluoroprobe will lead to many innovative "on-site" bio-analytical applications.

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VITA

Bruce Jason Tromberg was born in Cincinnati, Ohio on November 25, 1957. He received a Bachelor of Arts degree in Chemistry and Psychology from Vanderbilt University in May 1979. In June 1982, the author had the good fortune of marrying the former Patricia Watkins of Nashville, Tennessee. He received the Master of Science degree in Organic and Analytical Chemistry in December 1983 from The University of Tennessee, Knoxville.

Mr. Tromberg will be employed with Oak Ridge National Laboratory after graduation.