

Time Resolved Fluorescence Spectroscopy for in Situ Measurements¹

TORSTEN ALVAGER and MARK BRANHAM

Department of Physics, Indiana State University, Terre Haute, Indiana

Introduction

Fluorescence measurements of biological material in situ is a growing and potentially very powerful technique for many biological and medical studies. In most existing fluorometers adapted for in situ measurements a beam of excitation light is directed towards the sample position and fluorescence light is then detected in the fluorometer. In general, the method for creating a beam involves the use of a microscope in which a parallel beam of light is focused into a small spot at the sample position. This system is suitable for studying fluorescence from surface targets but is relatively complicated to use, especially if observations have to be performed over a longer period of time of living material. In such cases a more flexible arrangement to direct the light towards the sample spot is through the use of the recently developed light guide technique (5). This method may also allow fluorescence measurements of cells situated deep inside tissue and organs (2).

The most important example of in situ fluorescence measurement is probably the assay of oxidation-reduction state of NADH in which this compound serves as an indicator of the rate of oxygen consumption and the rate of ATP formation in a tissue (3). This method has been applied, for instance, in observation of NADH fluorescence as a measure of oxygen consumption in various changing states of the cerebral cortex of cats in situ (6).

A problem that confronts most in situ fluorescence studies is the effect of scattered and reflected excitation light. For a fluorophore with high quantum yield and suitable sample concentration (like NADH in the illustration mentioned above) it is often possible to separate, satisfactorily, the scattered light from the fluorescence light by the use of a monochromator or a filter. However, for less favorable cases spurious scattering light may dominate over fluorescence light even at the fluorescence wavelength and make a meaningful reading difficult or impossible. Under such circumstances, an improved fluorescence signal would be possible to obtain in many cases by separating the two light signals in time, since relative to the excitation light the fluorescence light is often delayed (often in the nanosecond range) while the scattered light is prompt. This possible method has been investigated in the present work. A nanosecond fluorescence spectrometer has been adapted to a light guide arrangement for observation of fluorescence and various experimental parameters of such a system have been measured.

¹Supported in part by a grant from the ISU Research Committee.

Method

A schematic block diagram of the experimental set-up is seen in Fig. 1. Nanosecond light pulses from an air spark source are selected in filter 1 and passed partially through a beam splitter. The light then enters a light guide system, consisting of a Schott uv-light guide having an effective diameter of 0.1 cm. The distal end of the light guide is in contact with the sample. Fluorophores in the immediate vicinity of the end point of the probe will be excited by the incoming light and re-emit some fluorescence light into the light guide. This light together with some scattered and reflected light is transported back to the beam splitter and part of it reflected into the detector system and registered after selection in filter 2. The time difference between light source pulses and corresponding detector pulses are measured in the time measuring unit which is a conventional delayed coincidence system and a time spectrum is finally obtained.

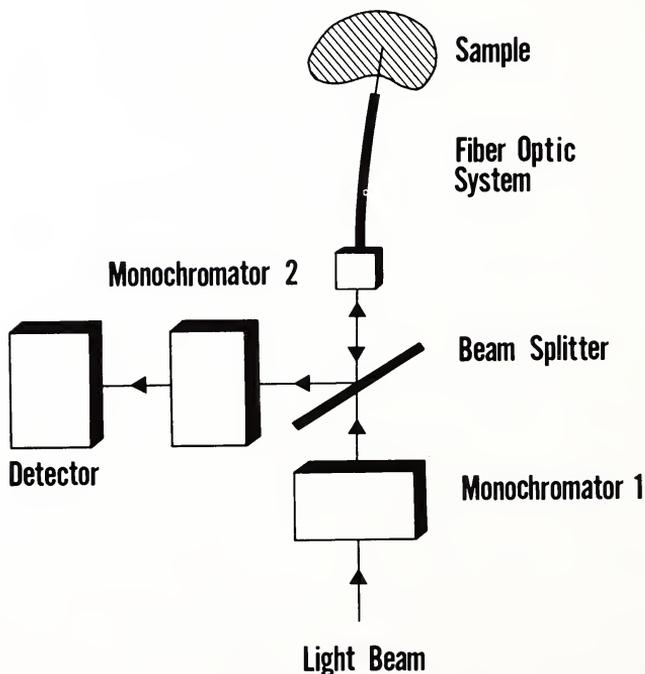


FIGURE 1. Block diagram of fiber optic fluorometer

The instrument outlined in Fig. 1 is usually referred to as a nanosecond fluorescence spectrometer (1). The main difference between the present set-up and a conventional instrument is the light guide system. Light emitted from the spark, maintained between two high voltage electrodes, is focused by a lens to the entrance surface of the light guide, which has a diameter of approximately 0.1 cm. The beam splitter consists of a mirror with a small opening (diameter = 0.05 cm) transmitting excitation light into the light guide. Part of the light returning

through the light guide is reflected by the beam splitter into the detector. The filters are interference filters. The detector is an RCA 8850 photomultiplier for registration of single photons.

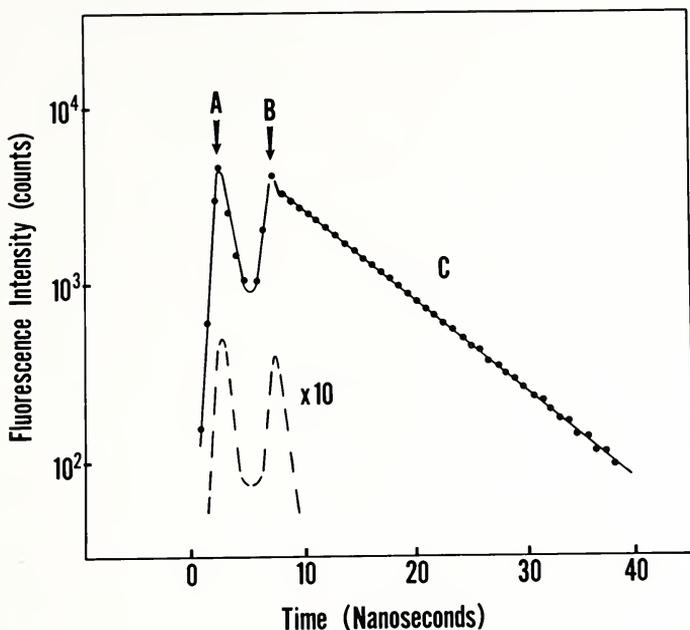


FIGURE 2. Time spectra obtained with the fiber optic probe shown in Fig. 1 of quinine sulfate fixed in gelatine (—●—●—●—) and pure gelatine (-----). Excitation at 350 nm; emission of 460 nm.

Results and Discussions

A typical time spectrum measurement with the light guide can be seen in Fig. 2, where the numbers of counts registered by the detector is plotted vs. delayed time (in nanoseconds). The sample was 5×10^{-5} M quinine sulfate in 0.1 N H_2SO_4 fixed in gelatine to simulate biological material. The two filters had maximum transmission at wavelengths 350 nm (filter 1) and 460 nm (filter 2) respectively, corresponding to optimal excitation and emission conditions in quinine sulfate. The two peaks in Fig. 2 are due to a small residue of scattered and reflected light which, in spite of the filters, reached the detector. Peak A corresponds mainly to excitation light scattered into the detector from the section around the beam splitter, while Peak B is due to light passed through the light guide and scattered or reflected in the sample. The time difference between the two peaks is due to the time of flight of light in the light guide. The continuous part of the curve (C) in Fig. 2 corresponds to fluorescence light from quinine sulfate. The slope of the decay curve gives the lifetime of the decay. In this case it is 10.5 ns. This value can be compared to the lifetime of quinine sulfate in pure 0.1 N H_2SO_4 , which is 19.0 ns (4). The smaller value of the lifetime in the present case is mainly due the environmental change caused by the presence of the gel.

The data presented in Fig. 2 shows clearly the advantage of using a timing device to separate fluorescence light from scattered light. The degree of separation depends, of course, on the lifetime of the fluorophore and the time resolution given by the instrument. In the present case the fluorescence lifetime (10.5 ns) is much longer than the instrumental time resolution (approximately 2 ns) and the separation is good. For shortlived fluorophores several factors are of importance in determining the degree of separation. Of special interest for the light guide technique is the spread in time due to different paths of light rays in the light guide. To study this problem in more detail Fig. 2 gives also a measurement with a quinine sulfate absent from the sample (dashed curve) to enhance the prompt peaks. In this experiment two light guides were used: one situated between the beam splitter and the sample and one between the beam splitter and the detector. Both light guides were approximately 0.5 m in length. Light giving rise to peak A therefore traversed a distance three times shorter than light responsible for peak B. In spite of the length difference, the widths of the two peaks are approximately equal, which means that the time spread in the light guides contributes to less than 1 ns of the peak widths. For lifetimes in the range 1-2 ns this spread may be of importance. However, in such critical situations shorter light guides than those employed here may be used.

It should be noted that by an improved design of the system the intensity of the scattered light could be reduced. For instance, the use of high quality monochromators instead of filters would attenuate the intensity of scattered and reflected light considerably. However, with the particular geometry necessary to use in connection with the light guide, non-fluorescence light will always be present and will give rise to some residual light that can enter the detector. The time measurement procedure will therefore be of importance in most situations, especially for fluorophores with a low quantum yield.

Literature Cited

1. ALVAGER, T. and W. X. BALCAVAGE, 1974. Nanosecond fluorescence decay study of mitochondria and mitochondrial membranes. *Biochem. Biophys. Res. Comm.* **58**:1039-1046.
2. ALVAGER, T., 1977. Microfluorometry with optical fiber microprobe. To be published.
3. CHANCE, B., P. COHEN, F. JOBSIS, and B. SCHOENER, 1974. Intracellular oxidation-reduction states in vivo. *Science* **137**:449-508.
4. GUILBAULT, G., 1973. *Practical Fluorescence*. Marcel Dekker, Inc. New York, p. 13.
5. MAVESKY, A. and B. CHANCE, 1973. A new long-term method for the measurement of NADH fluorescence in intact rat brain with chronically implanted cannula. *Oxygen Transport to Tissue*: 239-244.
6. ROSENTHAL, M. and G. SOMJEN, 1973. Spreading depression, sustained potential shifts, and metabolic activity of cerebral cortex of cats. *J. Neurophysiol.* **36**:739-749.