ment of this approach is just now beginning. Although it is promising in principle, it is still too soon to decide whether sufficiently accurate measurements can be obtained over a wide enough range of conditions to yield the desired information.

It does not seem likely that FCS will be much used to study conformational fluctuations. Optical changes associated with conformational transitions are typically too small for successful application of this approach. Application of FPR to this kind of problem would require that particular conformational isomers be specifically sensitive to photolysis. This considerably narrows the choice of acceptable systems. A different approach would be to measure the relaxation of very small displacements from equilibrium generated by small perturbations of state variables such as temperature and pressure. Although those would be forced displacements, they could approach spontaneous fluctuations in magitude. Since the optical signals generated by such small changes in state are also very small, it is necessary to perturb and measure repetitively with signal averaging to achieve adequate precision. A method of this kind, based on small repetitive pressure jumps, has been developed.[56,57] Perturbation magnitudes are typically 10- to 100-fold smaller than in corresponding temperature- or pressure-jump experiments in which only one or a few transient relaxations are observed.

Acknowledgments

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[56] R. M. Clegg, E. L. Elson, and B. W. Maxfield, *Biopolymers* **14,** 883 (1975).
[57] H. R. Halvorson, *Biochemistry* **18,** 2480 (1979).

[20] Fluorescence Lifetimes with a Synchrotron Source

By STEVEN G. BOXER and RODNEY R. BUCKS

Synchrotron Radiation for Timing Experiments

Synchrotron radiation is a unique light source for use in the measurement of fluorescence phenomena. The light pulses generated by an electron storage ring cover all wavelengths of interest to spectroscopists working with biological molecules, have a high repetition rate, and are

immune to shot-to-shot fluctuations in intensity. While mode-locked lasers can produce shorter pulses with greater pulse powers, they do not have the ease of excitation tunability of a synchrotron source. If photon-counting techniques are used, the low pulse power of synchrotron sources is not an important consideration. Visible light from the synchrotron had been used routinely by operators at the Stanford Synchrotron Radiation Laboratory (SSRL) for luminosity measurements and position adjustments. The notion of intercepting a fraction of this beam for lifetimes measurements was conceived by Drs. Ian Munro and Andrew Sabersky.[1]

The fluorescence lifetimes port at SSRL utilizes light pulses generated by electrons stored in the Stanford Positron Electron Asymmetric Ring (SPEAR). Although similar capabilities are available at other synchrotron sources, the authors' experience is limited to SSRL. The reader interested in other synchrotron sources should consult the abstracts of the 1983 Meeting of the American Society for Photobiology where these sources are compared.[2] As the characteristics, advantages, and problems associated with the measurement of fluorescence lifetimes at SSRL are typical, we will only discuss this facility.

The minimum repetition rate of the light pulses is 1.28 MHz, which corresponds to a single bunch of electrons circulating in the ring. This minimum repetition rate is determined by the ratio of the speed of light and the ring circumference. The total number of bunches of electrons which can be stored in SPEAR is 280; this value depends on the frequency of the accelerating rf field used to supply energy to the electrons as they circulate (358 MHz in SPEAR).

In practice, a pattern consisting of a small number of electron bunches ($\leqq$17) is stored in the ring. At any time this number is determined by a compromise among the needs of the many users of radiation emitted by the synchrotron source. The simplest pulse configuration occurs during runs when colliding beam experiments (electrons and positrons) are performed by high energy physicists. A single electron bunch (20–30 mA) circulates in the ring; this gives rise to light pulses which are separated by ~781 nsec. Due to the geometry of the optics the lifetimes port only receives light from the stored electrons, not the positrons. Another common mode of operation is the "timing" mode, where 4 or 5 single bunches of electrons are spaced roughly equally around the ring. The time between light pulses is 195 nsec (4 bunches) or 156 nsec (5 bunches). In order to

[1] I. H. Munro and A. P. Sabersky, *in* "Synchrotron Radiation" (H. Winick and S. Doniach, eds.), p. 323. Plenum, New York, 1980.

[2] *Abstr. Annu. Meet. Am. Soc. Photochem. Photobiol., 11th* **37**, 525–529 (1983).

obtain the highest energy radiation (X rays), highest intensity, and long beam storage time, a common mode of operation is four groups of four adjacent electron bunches, roughly equally spaced (4 × 4). Within the groups of four, the bunches of electrons are separated by 2.79 nsec. This mode is useless for fluorescence lifetimes as the pulse spacing within the groups of four is much too short. This can be circumvented by injecting a seventeenth electron bunch between two of the groups of four (4 × 4 + 1); the single bunch is isolated from any group of four by ~89 nsec. It has been found that this does not degrade overall performance. The electronics can be adjusted so that only fluorescent events caused by the light pulse from the single electron bunch are recorded, even though the detector sees events generated by each pulse. Of course, if the fluorescence lifetime of the sample is long (say longer than one-fifth of the interpulse spacing), some of the above configurations may not be suitable.

Under typical operating conditions of SPEAR (3.0 GeV, 5–10 mA in the timing electron bunch) the electron bunch and therefore the light pulse has a full width at half maximum of ~200 psec, although pulses as short as 55 psec are possible.[1] The pulse is roughly Gaussian in temporal profile, but is not perfectly so. Typically, there are about 10^4 photons per pulse (4 nm band width). Sabersky has studied the details of the pulse substructure using a streak camera.[3] Depending on the precise SPEAR operating conditions irregular pulse shapes and shape changes have been observed. These irregularities could affect the accuracy of measurements of very short fluorescence lifetimes; no systematic study has yet been undertaken.

The lifetimes port is located in a large freight container on top of the radiation shielding of the storage ring as shown in Fig. 1. The light is generated in one of the SPEAR bending magnets; it is reflected from a Be mirror and passes out of the beam vacuum chamber through a 1-cm Suprasil window. A front surface aluminized beam splitter (50–90%) reflects the light beam upward to the lifetimes port where an adjustable mirror directs the beam horizontally. The dimensions of the beam after reflecting from the mirror in the lifetimes port are about 1 × 5 cm (roughly rectangular). The optical components and ambient atmosphere determine the available wavelength range in the port (longer than 200 nm). With standard monochrometers, narrow bandwidth excitation can be obtained over the entire ultraviolet, visible, and infrared range. Experiments at higher energy (vacuum ultraviolet, soft X ray, etc.) require special high-vacuum systems and are available at other SSRL beam lines. The beam is highly linearly polarized in the vertical direction (Fig. 1).

[3] A. P. Sabersky and M. H. R. Donald, SLAC-PUB-2696, PEP-NOTE-350, February 1981.

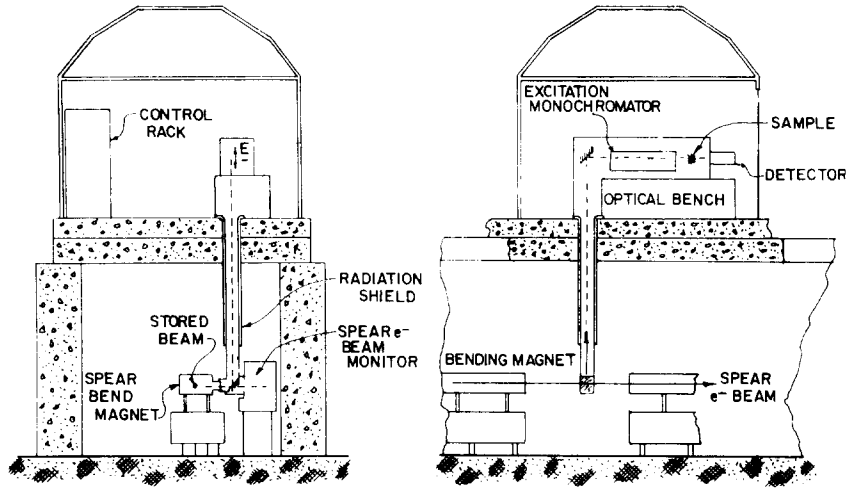

FIG. 1. An illustration of the fluorescence lifetimes port beamline at SSRL. The light beam is reflected from a Be mirror in the vacuum chamber, passes out through a Supracil window, and is reflected upward into the shack containing the sample and electronics.

Time-Correlated Single-Photon Counting

The theory of time-correlated single-photon counting has been discussed in many places and the interested reader is referred to the article by Yguerabide[4]; only a brief description of the important points will be given here. A fluorescent sample is irradiated with a short pulse of light; the amount of time which elapses between excitation and the detection of the first fluorescent photon is recorded. The data are a histogram of the number of fluorescent events which occurred at a given time after excitation and correspond to the fluorescence decay. Because of the recovery time and design of the electronics, one photon or less per light pulse is detected. This means that any fluorescent photons which strike the photocathode of the photomultiplier tube after the first photon will not be detected. If the accumulated data are to accurately represent the true fluorescence decay, it is necessary to avoid the situation where two or more fluorescent photons strike the photocathode per excitation pulse. Because only the first photon is detected, this would weight the decay toward shorter times. The simple solution is to count events with a low efficiency in terms of the number of fluorescent events detected compared to the number of light pulses which impinge on the sample. It has been shown that a detection rate of less than 2% of the sample excitation rate will lead to a measured decay which has less than 1% distortion relative to

[4] J. Yguerabide, this series, Vol. 25, p. 498.

the true decay.[4] Therefore, counting rates at SSRL are maintained lower than 25 kHz to avoid distortion of the data.

Instrumentation

The light from the horizontal mirror is focused onto the entrance slit of a monochromater which allows the selection of narrowband excitation. A number of grating sets are available for the monochrometer (e.g., 300, 750, and 1000 nm blaze) and the gratings are easily interchangeable (SPEX Doublemate). The slit width can also be adjusted. Upon exiting the monochrometer the light beam is directed onto the fluorescence sample, and fluorescence is detected at 90° to the direction of the excitation beam. Several photomultiplier tubes (PMTs) have been used, including Hamamatsu R1333 and RCA 8850 and 8852. The fluorescence is typically filtered with colored glass or interference filters to eliminate scattered light or it can pass through another monochrometer. The spread in the bunch of photoelectrons as it moves from the photocathode down the dynode chain (transit time jitter) typically broadens the observed pulse width from ~200 to ~600 psec.

A block diagram of the electronics is shown in Fig. 2; this is typical of photon-counting systems.[5] The heart of the system is the time-to-amplitude converter (TAC). The TAC accepts a start and stop pulse and produces an output voltage which is proportional to the time which elapses between these two pulses. At SSRL the start signal is initiated by a photon impinging on the photocathode of the PMT. The PMT tube output then goes to a constant fraction discriminator (CFD), and the CFD output is the start pulse for the TAC. It is necessary to use a constant fraction discriminator rather than a leading edge discriminator for this application because of the amplitude fluctuations of the PMT output pulse. The timing characteristics of the CFD are not dependent on pulse amplitude. The stop pulse originates in a signal from an rf electrode placed in the beam chamber which generates a signal as the electron bunch passes. The rf electrode signal is input to the Three Flavor Beam Trigger (TFBT). The TFBT is a discriminator which was built at the Stanford Linear Accelerator Center for use in timing applications on another storage ring. It is designed to respond to the fast, bipolar rf electrode pulse with little time walk as the pulse amplitude decays with time (due to inevitable degradation of the electron beam current). The output from the TFBT is input to a 100 MHz leading edge discriminator which produces the stop pulse.

Because there may be more than a single electron bunch in the storage ring (e.g., $4 \times 4 + 1$), it is necessary to reject data which are not due to the

[5] K. G. Spears, L. E. Cramer, and L. D. Hoffland, *Rev. Sci. Instrum.* **49**, 255 (1978).

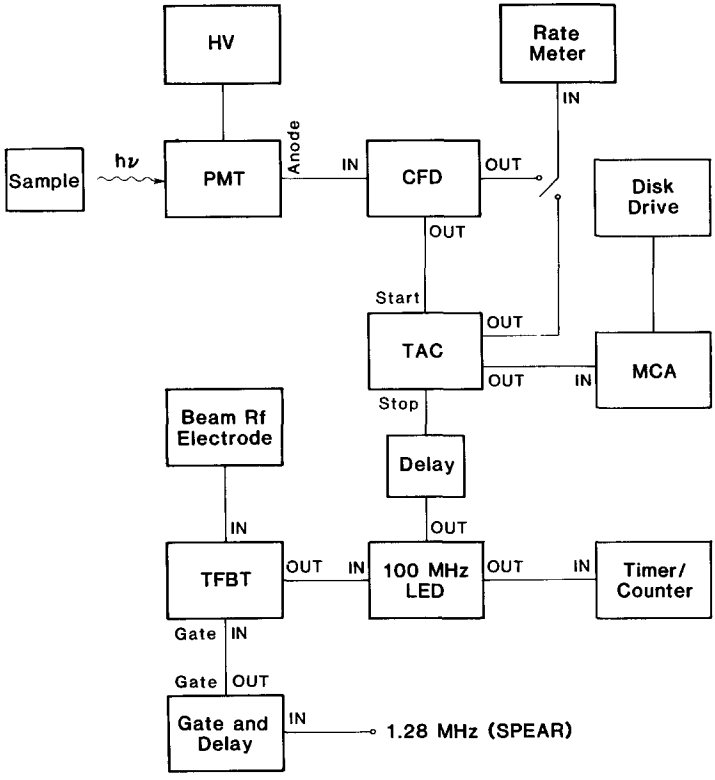

FIG. 2. Schematic diagram of the electronics used for time-correlated single-photon counting. CFD, Constant fraction discriminator; HV, high voltage; LED, leading edge discriminator; MCA, multichannel analyzer; PMT, photomultiplier tube; TAC, time-to-amplitude converter; TFBT, three flavor beam trigger.

single bunch. This is accomplished by gating the TFBT; it only produces an output pulse when the gate is activated. A 90-nsec gate pulse is produced by a gate and delay generator and supplied to the TFBT. A 1.28 MHz signal from the SPEAR master oscillator is used as the input to the gate and delay generator. The delay of the gate and the delay generator can be adjusted so that the TFBT is active for any 90 nsec within the 781-nsec cycle period of SPEAR. By adjusting this delay, the stop signal can be derived from the single bunch of electrons or any other of the electron bunches. The TAC only produces an output voltage pulse when it receives both a start and a stop signal. By gating the stop signal, fluorescent events which are detected by the PMT, but were caused by other than the desired electron bunch, can be ignored.

The TAC produces an output voltage which is proportional to the time interval between the start and stop pulses, and this is input to a Multi-Channel Analyzer (MCA), run in pulse height analysis mode. The MCA records the number of times that the TAC produced a given voltage by placing a count in channel n which corresponds to the voltage $n\Delta V$. ΔV is the voltage interval per channel of the MCA. The voltage is proportional to a time interval, and therefore the channel corresponds to an elapsed time between detection of a fluorescent photon (start) and the timing signal (stop). Because the timing signal is fixed in time with respect to the excitation pulse (both are derived from the circulating electron bunch) each channel of the MCA data contains the record of the number of fluorescent events detected at time $n\Delta t$ ($\Delta t \propto \Delta V$) after excitation. This is the fluorescence decay.

Although the pulse-to-pulse stability with a synchrotron source is extraordinary, the circulating electron current slowly decays over a period of hours. A separate photomultiplier has been provided which measures the exciting beam intensity as a function of time for data scaling during very long-term averaging.

In normal operation both a scattered light pulse profile (e.g., scattered light from coffee creamer) and a fluorescence decay are collected for each sample. Because the time response of the PMT varies with the wavelength of incident light, it is important to collect the pulse profile data at the wavelength that the sample emits. This is easily done by setting the excitation monochrometer at that wavelength (this calibration is easy to perform given the tunability of the light; this can be a source of considerable experimental difficulty with dye laser excitation). The temporal profile of synchrotron radiation is invariant with respect to wavelength, an important advantage of synchrotron radiation. The arrival time of each wavelength will vary slightly because of the intervening optics.

If the MCA is operated in multichannel scaling mode (MCS), time-resolved fluorescence excitation spectra can be obtained. This is possible because synchrotron radiation acts as a white light source, and the excitation can be scanned with a monochrometer. In MCS mode the MCA records the frequency of occurrence of events versus elapsed time. By using the upper level and lower level discriminators to set the limits of a voltage range (therefore time range) on the MCA, the MCA will record the number of events occurring as a function of time which have a voltage greater than the lower level setting and lower than the upper level setting. If the upper and lower level discriminators are set while the MCA is in pulse height analysis mode, one can see that a region of the decay can be selected which corresponds to events which occur at a given time with respect to excitation. If the MCA (in MCS mode) and excitation mono-

chrometer are scanned simultaneously, the MCA records an excitation spectrum of events which occur during the selected time interval of the decay. The convenience of doing this type of experiment with a readily tunable source cannot be overstated.

A nonlinear least-squares routine is used to fit exponential decays to the fluorescence data. The method is known as the linearly constrained modified diagonal method and is based on a program originally written at the National Institutes of Health.[6,7] The program will fit a single exponential or the sum of exponential decays to the data. Many more or less sophisticated versions of software for deconvolution have been described; several are commercially available.

Performance

The major timing limitations in the experimental set-up are the response time and timing jitter of the PMT and time walk of the timing signal (stop pulse). The time jitter of the electronics is small (<50 psec) and this adds little to the observed pulse width, while the jitter in the PMT response broadens the measured pulse profile from the actual ~200 to ~650 psec. The addition of the TFBT solves the timing pulse walk problems. The rf electrode picks up the signal from the electron bunch ~3 m from the light source in the bending magnet and is directly beneath the lifetimes port. Because both the light pulse and rf electrode signals are generated directly from the beam, the timing pulse should not walk in time. The TFBT is insensitive to changes in signal amplitude as the beam decays, and produces a very stable timing signal. The 1.28 MHz master oscillator signal cannot be used as the timing pulse. The light pulse arrival time varies with respect to the 1.28 MHz signal as the beam decays and as the phase of the 358 MHz rf cavities are adjusted or drift.

The fluorescence lifetimes of several compounds with known lifetimes were used to test the capabilities of the facility. The measured fluorescence lifetimes of a chlorophyllide dimer in CH_2Cl_2 (100 ± 30 psec),[8] Rose Bengal in methanol (520 ± 50 psec),[5] and Rhodamine 6G in ethanol (3.8 ± 0.2 nsec)[9] all agree with the reported lifetimes within the quoted experimental error. There is evidence that the longitudinal structure of the electron bunch changes with time under certain operating conditions,[3]

[6] R. I. Schrager, *J. Assoc. Comp. Mach.* **17,** 446 (1970).

[7] A. Grinvald and I. Z. Steinberg, *Anal. Biochem.* **59,** 583 (1974).

[8] M. J. Pellin, M. R. Wasielewski, and K. J. Kaufmann, *J. Am. Chem. Soc.* **102,** 1868 (1980).

[9] D. R. Lutz, K. A. Nelson, C. R. Gochanour, and M. D. Fayer, *Chem. Phys.* **58,** 325 (1981).

and this will interfere with the measurement of fluorescent lifetimes which are comparable to the pulse width.

The high repetition rate of the excitation pulse at SPEAR allows the rapid accumulation of fluorescence decays with good signal-to-noise. Accumulation times of 5 min were typical for samples which had a fluorescence quantum yield of 0.3, absorption at the excitation wavelength of 0.05, and an excitation band pass of 5 nm. Low sample optical densities are required to prevent artificial lengthening of the fluorescence lifetime due to emission–reabsorption.

Several applications have been described in the literature. Monro, Pecht, and Stryer examined the fluorescence lifetimes and fluorescence anisotropy decay for a number of small proteins each containing a single tryptophan residue.[10] The combination of high intensity excitation light in the near ultraviolet (280 nm) and the highly polarized beam gave very good signal-to-noise and permitted a comparison of the degree of motional reorientation of tryptophan in these proteins. Our group has studied fluorescence lifetimes from a wide range of chlorophyll derivatives,[11,12] synthetic chlorophyll–protein complexes,[13] and photosynthetic reaction centers.[14] In the case of synthetic aggregates of chlorophyll-type chromophores, multiple decays are quite common. It is very important to distinguish decays due to impurities which may have a very high fluorescence quantum yield from real components of interest. The ability to record time-resolved excitation spectra has proved most useful in discriminating impurity fluorescence from the desired decay components. It is straightforward to scan the excitation monochrometer from 300 to 700 nm and compare the spectra giving rise to the fastest and slower decaying components.[12] Although such an experiment is possible in principal with a synchronously pumped dye laser system, it is a major undertaking, involving many different laser dyes and enormous variations in intensity over such a wide spectral range.

At the present time there have been relatively few applications of this technique to biological problems. This is undoubtedly because of the false impression that synchrotron experiments are exotic and inaccessible to the nonspecialist. The facility at SSRL is available for general use and is no more complex to operate than a commercial lifetimes apparatus. A

[10] I. Monro, I. Pecht, and L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 56 (1979).
[11] R. R. Bucks and S. G. Boxer, *J. Am. Chem. Soc.* **104,** 340 (1982).
[12] R. R. Bucks, I. Fujita, T. L. Netzel, and S. G. Boxer, *J. Phys. Chem.* **86,** 1947 (1982).
[13] S. G. Boxer and K. A. Wright, *Biochemistry* **20,** 7546 (1981).
[14] C. E. D. Chidsey, Ph.D. thesis, Stanford University (1983).

new facility at the Brookhaven National Laboratory is likely to be even more flexible and equally useful for experiments in the ultraviolet region.

Acknowledgments

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[21] Fluorescence Polarization at High Pressure

By ALEJANDRO A. PALADINI, JR.

Introduction

For many years pressure has been used as a key variable in gathering information on the structure of matter. About a hundred years ago the effect of pressure on living systems was reported for the first time by two French scientists, P. Regnard and A. Certes.[1] Since then it has become quite common to submit living organisms to pressure. The results obtained in those early efforts show that the general aim was to prove that pressure had an effect (usually negative) on the systems studied rather than its use as a thermodynamic variable.

The reader interested in a review of the literature dealing with high-pressure effects upon biological systems is directed to those recently published by Morild[2] and by Heremans.[3]

However, the assumptions, limitations, and consequences of the theory employed so far in the interpretation of experimental data can be found in a review by Weber and Drickamer.[4] Hydrostatic pressure is a scalar magnitude and as such it is expected to act without preferential direction on the media to which it is applied. Furthermore, the use of pressure permits the study of effects related exclusively to changes in volume. This is not generally possible when the temperature and/or chemical composition of the system has been selected as the disturbing

[1] P. Regnard, "Recherches Experimentales sur les Conditions Physiques de la Vie dans les Eaux." Librairie de L'Academie de Medicine, Paris, 1891.
[2] E. Morild, *Adv. Protein Chem.* **34,** 93 (1981).
[3] K. A. H. Heremans, *Annu. Rev. Biophys. Bioeng.* **11,** 1 (1982).
[4] G. Weber and H. G. Drickamer, *Q. Rev. Biophys.* **16,** (1983).