Fluorescence dynamics of biological systems using synchrotron radiation

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A beamline for time-resolved fluorescence spectroscopy of biological systems is under construction at the Synchrotron Radiation Center. The fluorometer, operating in the frequency domain, will take advantage of the time structure of the synchrotron radiation light pulses to determine fluorescence lifetimes. Using frequency-domain techniques, the instrument can achieve an ultimate time resolution on the order of picoseconds. Preliminary experiments have shown that reducing the intensity of one of the fifteen electron bunches in the storage ring allows measurement of harmonic frequencies equivalent to the single-bunch mode. This mode of operation of the synchrotron significantly extends the range of lifetimes that can be measured. The wavelength range (encompassing the visible and ultraviolet), the range of measurable lifetimes, and the stability and reproducibility of the storage ring pulses should make this beamline a versatile tool for the investigation of the complex fluorescence decay of biological systems.

I. INTRODUCTION

Fluorescence spectroscopy has been established as one of the major physical methods to investigate biological molecules, providing detailed information of structural and dynamic properties of macromolecules. A variety of fluorescence techniques have been used to investigate all the major components of cells: proteins, nucleic acids and membranes.[1] In particular, the study of the fluorescence decay and the decay of the emission anisotropy provides information on the chemico-physical environment of macromolecules and their dynamics. Fluorescence methods are now also being used in biomedical areas where sensitivity and specificity are crucial requirements. These important applications are necessarily dependent upon the study of the basic fluorescence processes.

The measurement of the fluorescence decay can be obtained by two alternative methods: 1) in the time domain, where the decay is recorded using the technique of correlated single photon counting; 2) in the frequency domain, where the harmonic response is measured. Having time structure with harmonic content in theGHz region and exact timing of the pulses make an electron storage ring an ideal source for fluorescence studies in the frequency domain. In addition, its continuum spectrum provides excitation energies in the UV that are not accessible with high repetition pulsed lasers.

The first frequency-domain fluorometer was installed in the Frascati Synchrotron in 1983[2] followed by a dedicated beamline ("Plastique") for studying the fluorescence of biological materials using the frequency domain method.[3] In the present paper we describe a new beamline near completion at the Synchrotron Radiation Center (SRC) constructed for this purpose.

II. OPTICAL DESIGN

The design goals for the fluorescence lifetime beamline were to focus the photon beam to a size smaller than the normal entrance slit settings of the monochromator (1 mm wide by 8 mm high) and match the acceptance of the f/4 monochromator. The beamline was assigned port 073 of the Aladdin storage ring. This is an inside port blocked by adjacent beamlines, and the accessibility to this port is very poor. However, the optical design is simplified by the wavelengths requirements, from the visible down to 200nm, which allows one to use large deflections.

The optical layout of the beamline is illustrated in Fig. 1. The first optical element, M0, is a spherical mirror with a 1.5-m focal length collecting 40 mrad horizontally and the full fan of radiation in the vertical plane. M0 deflects the

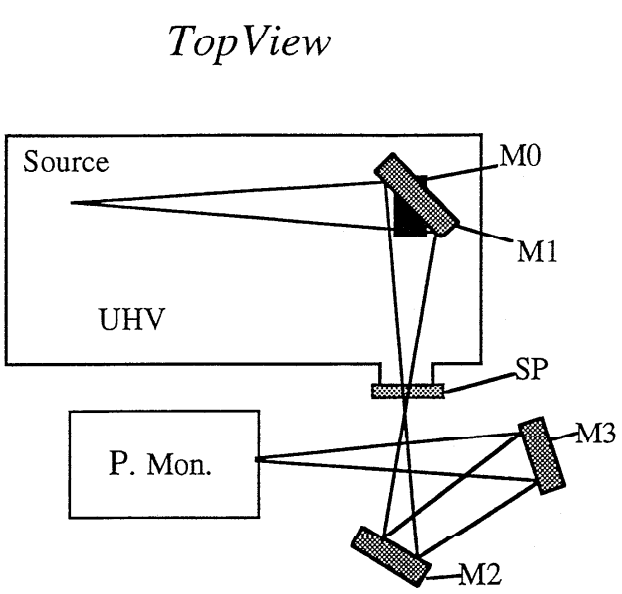

FIG. 1. Optical layout of the fluorescence beamline. M0 and M1: 3-m-radius spherical mirrors; SP: sapphire window; M2: plane mirror; M3: 0.4-m-radius spherical mirror; P. Mon: UV-VIS monochromator.

light upward by 90 degrees to clear the ring and adjacent beamlines. This mirror has a copper heat sink attached to the back, which is water cooled. The mirror is mounted from the front surface to minimize heat effects on the actual reflecting surface. The combination of front-surface mounting and cooling is expected to give stable performance for all anticipated future power levels. The large angle of incidence ensures that M0 absorbs all the unwanted high energy radiation. Based on its high reflectivity in the energy range required by the experiments and on its thermal stability, we opted for platinum as the reflecting coating. We note in passing that carbon contamination in the optic can be cleaned from a platinum optical coating.[4] The second optical element (M1) is also a spherical mirror with a 1.5-m focal length that deflects the light 90 degrees perpendicular to the plane of the first reflection. Since no significant heat load is incident on M1, this element is coated with aluminum with an overcoating of MgF_2,[5] a coating that provides the highest reflectivity in the UV wavelength region. The perpendicular reflection planes of M0 and M1 cause the astigmatism of the two mirrors to cancel. The two-mirror system produces a unit magnification image of the synchrotron source. The image is rotated by 90 degrees as required by the vertically oriented monochromator slits.

A c-cut sapphire window located a few cm before the final image of the mirror system terminates the ultrahigh vacuum part of the beamline. The following two optical elements, a plane mirror (M2) and a spherical mirror with a 0.2-m focal length (M3) are in air. Both are coated with UV-enhanced aluminum. They redirect the light from the intermediate image and refocus it onto the entrance slit of the monochromator. Ray tracings of the optical system with the SHADOW package[6,7] show that the focused beam size is smaller than the entrance slit and the grating is almost fully illuminated.

III. EXPERIMENTAL TECHNIQUE

A. Phase Fluorometry and Cross-Correlation

The theory of phase fluorometry using a single frequency was described by Dushinsky.[8] Briefly, a fluorescent sample is excited with light modulated at an angular frequency ω

$$E(t) = E_0[1 + M_E \sin(\omega t + \phi_E)], \tag{1}$$

where M_E is the modulation factor, t is the time, and ϕ_E the phase. In the particular case in which the fluorescence is due to a single exponential decay $\exp(-t/\tau)$, where τ is the lifetime, the emission can be expressed as

$$F(t) = F_0[1 + M_F \sin(\omega t + \phi_E - \phi)], \tag{2}$$

where

$$\tan\phi = \omega\tau \text{ and} \tag{3}$$

$$\cos\phi = M_F = [1 + (\omega\tau)^2]^{-1/2}. \tag{4}$$

Evidently, the decay lifetime can be obtained by measuring the phase or from the modulation of the fluorescence M_F. The latter can be obtained experimentally from the alternating (AC) and direct (DC) terms of the signals, i.e.,

$$M_F = (AC/DC)_F / (AC/DC)_E. \tag{5}$$

For systems emitting with multiple exponentials, the phase and the modulation are given by longer algebraic expressions.[9,10] The determination of the lifetimes in such cases requires measurements performed at more than one modulation frequency.

The addition of cross correlation techniques in phase fluorometry[11] allows measurements of the phase and modulation with high accuracy, low noise and better sensitivity. Moreover, as detailed below, this combination is the basis for phase fluorometry using a high repetition pulsed source. The principle of this technique consists of multiplying the output of the fluorescence and that of a reference by

$$C(t) = C_0[1 + M_C \sin((\omega + \Delta\omega)t + \phi_C)], \tag{6}$$

where C_0 is the DC component, M_C is the modulation ratio, $\Delta\omega$ is a very small angular frequency as compared to ω, and ϕ_C is the phase delay. From equations 1 or 2 and 6, it can be easily realized that the product signals contain a constant term, one with frequency $\Delta\omega$ as well as terms with frequencies ω and 2ω. The terms with $\Delta\omega$, which can be easily filtered from the rest, contain the original phase (with a sign change) and modulation information.

B. Phase Fluorometry and Cross-Correlation with Synchrotron Radiation

The use of a high repetition pulsed light source for multifrequency phase fluorometry was proposed by Gratton and Lopez-Delgado.[12] The conceptual basis for exploiting the time structure of synchrotron radiation in time-resolved fluorescence is based on the use of the harmonic content of the synchrotron radiation pulse.[2] Namely, the excitation of a system with a pulsed source can be seen as the combined excitation by all the harmonics contained in the pulse. The synchrotron radiation pulse is quasi-Gaussian with a width dependent upon the radio frequency used in the storage ring. For the Aladdin storage ring, the full width at half maximum of the pulses is about 900ps, and the pulse separation is approximately 19.8 ns when the ring is filled with 15 bunches (Fig. 2a). The corresponding power spectrum, shown in Fig. 2b, illustrates that the useful frequency range extends up to 1 GHz.

A scheme of the experimental setup for phase fluorometry using cross-correlation techniques to be implemented at Aladdin is shown in Fig. 3. For simplicity, we show only one cross-correlation step. The actual experimental setup contains two steps to improve the signal to noise ratio. The light pulses emitted from the storage ring are monochromatized by the UV-VIS monochromator. The monochromatized radiation is split by a beam splitter into two beams. One is directed to the reference photomultiplier (PM) and the other to the sample. The fluorescence signal from the sample is dispersed by the secondary monochromator and detected by a PM identical to the one employed to record the reference. The electronic circuit, described in section IIIC, splits the output of the PMs into their DC and AC components. The master oscillator, delivering the RF frequency (Ω– 50.5821MHz) to the storage ring, is used as a reference for the local oscillator. The local oscillator can be tuned to either Ω or its higher harmonics, plus 40 Hz. The latter is the cross-correlation frequency. The output of the local oscillator, shown in Fig. 3a for 2Ω+40 Hz, is mixed with the AC component of the output of the PM detecting the reference signal (Fig. 3b) and with that of the fluorescence signal (Fig. 3c).

Fig. 4 illustrates the application of the cross-correlation technique in phase fluorometry using synchrotron radiation. The result of mixing the AC component of the PM detecting the reference signal (Fig. 4a) with the cross-correlation signal (Fig. 4c) is shown in Fig. 4d. The mixing of the cross-correlation signal and the AC component of the PM detecting the fluorescence signal (Fig. 4b) is illustrated in Fig. 4e. As in Fig. 3 we assumed that the local oscillator is tuned to 2Ω plus 40 Hz and that the fluorescence signal is due to a single exponential decay representing a lifetime τ. As seen in Figures 4d and 4e, the resulting signals are modulated at the cross-correlation frequency, 40 Hz. The electronic system filters out from these signals all the high frequency

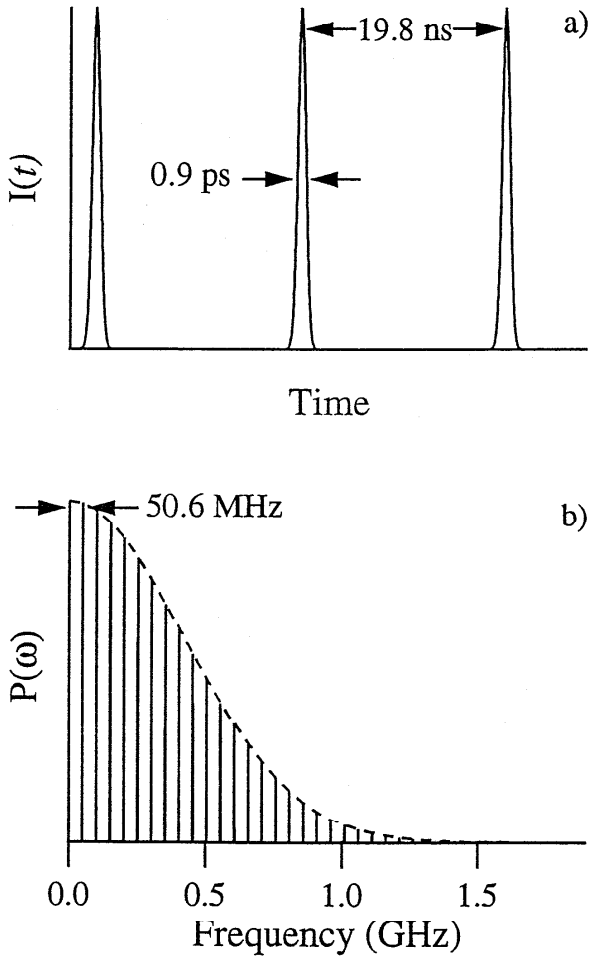

FIG. 2. (a) Time structure of the light pulses emitted from Aladdin with 15 bunches in the machine. (b) Fourier transform of a).

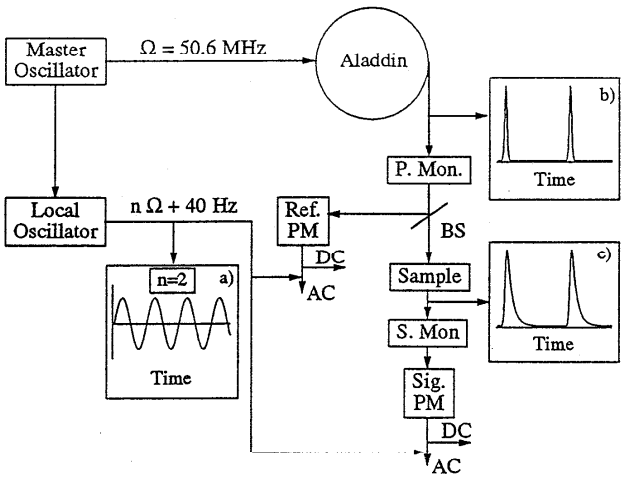

FIG. 3. Scheme of the experimental setup for phase fluorometry: P. Mon: primary monochromator; BS: beam splitter; Ref. PM and Sig. PM: reference and signal photomultipliers; S. Mon: secondary monochromator for dispersing the fluorescence. (a) Cross-correlation signal. (b) Excitation pulses. (c) Fluorescence response.

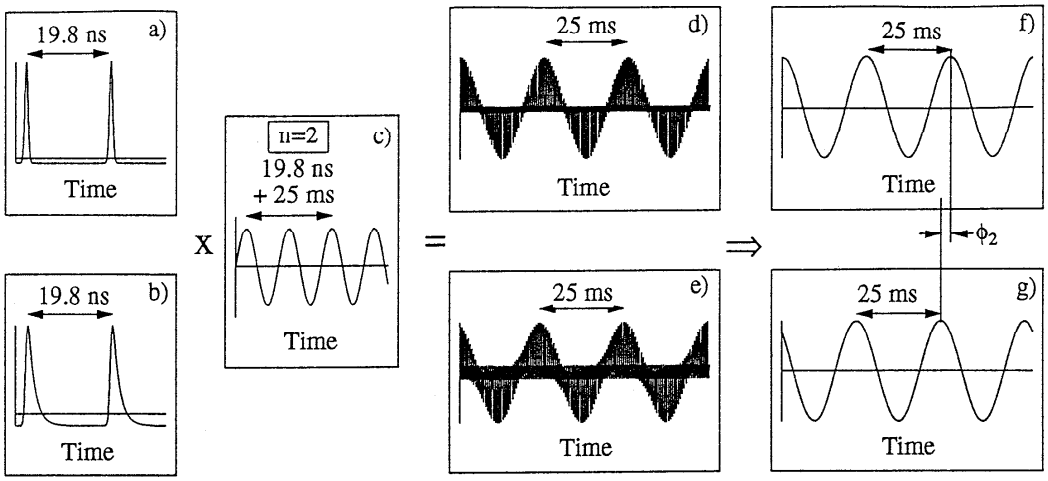

FIG. 4. Signal processing in phase fluorometry. (a) and (b) AC component of the reference and fluorescence PM output, respectively. (c) Cross-correlation signal. (d) Result of the mixing between a) and c). (e) Result of the mixing between b) and c). (f) Reference signal after filtering. (g) Fluorescence signal after filtering. ϕ_2: phase difference between f) and g). The zero line is indicated in all the graphs.

components besides the AC component at 40 Hz. Figures 4g and 4f present the result of the filtering for the reference and the emission signals, respectively. The phase difference shown in the figure, ϕ_2, is given by

$$\phi_2 = \arctan(2\pi 2\Omega\tau). \tag{7}$$

The lifetime, τ, can be also obtained from the filtered signals using the modulation ratio defined in Eq. 5, i.e.,

$$M_{F2} = [1 + (2\pi 2\Omega\tau)^2]^{-1/2} \tag{8}$$

The basic frequency separation of the harmonics depends on the repetition rate of the synchrotron radiation. In particular, for single-bunch mode operation at Aladdin, the minimum separation is 3.372MHz; whereas, when the maximum of 15bunches are injected in the ring, the minimum separation is 50.5821MHz. Due to the higher stored current in the ring, most of the experiments performed around the storage ring require the 15-bunch maximum. This mode of operation does not allow determination of lifetimes longer than a few tens of a ns using either single photon counting or phase fluorometry. As shown below, a novel method overcoming this difficulty in phase fluorometry has already been tested at Aladdin. In the frequency domain, the equivalent of a single bunch is to delete one bunch out of the 15 bunches. In practice, a reduction of the current in one of the bunches ensures the presence of all harmonic frequencies of the 3.3772MHz single-bunch fundamental. This simple, yet ingenious, maneuver permits full exploitation of the basic harmonic set of the Aladdin facility without interfering with normal operating conditions. It should be stressed that this approach is only valid in the frequency domain.

C. Electronic system

In practice, the frequency translation process is done in two steps. The scheme of the electronics for the superheterodyning detection is shown in Fig. 5 for one channel. The output of the PM (1 GHz bandwidth) is separated into its AC and DC components. Special care is used in this part of the circuit. Since this is the only microwave frequency part of all the signal processing, all components (capacitor, cables, connectors) must be rated for up to 1GHz operation. After the microwave mixer, M1 (Anzac, model MDC123), a frequency component of the signal is at low frequency (100KHz). It can then be handled using standard electronic

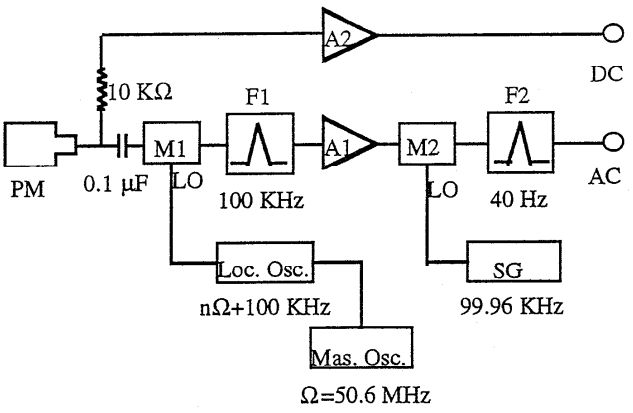

FIG. 5. Block diagram for one of the channels of the superheterodyning detection system. PM: photomultiplier. Loc. Osc.: microwave signal generator; Mas. Osc.: master oscillator providing the RF power to the storage ring; M1 and M2: double-balanced mixers with their respective local oscillator input signals (LO); F: narrow bandpass electronic filters for intermediate frequency of 100 KHz (F1) or cross-correlation frequency of 40 Hz (F2); A1: variable amplifier for AC signal; A2: DC signal amplifier; SG: signal generator.

components. It should be noted that the average DC current from the PM must not exceed 200nA, i.e., approximately 2V after the DC current-to-voltage converter (gain 10^7 V/A). About the same signal intensity with respect to the DC signal is available at each one of the harmonic frequencies over a spectrum that extends up to the limit imposed by the detector. However, the AC part of the signal has a 50 Ω impedance due to the mixer input characteristics, which typically results in a 10-μV signal. These signal levels are very low, and a direct conversion to the 40-Hz region is difficult, since the ubiquitous 60-Hz line signal dominates. This difficulty is one of the reasons for using the intermediate conversion frequency, where amplification can be obtained in a low noise frequency region. The mixer output is filtered using a 42IF301 ceramic IF filter transformer (Mouser Electronics, Mansfield, TX). This filter performs an impedance conversion from about 500 Ω to about 50K Ω, providing a voltage gain of about a factor of 100. The output of the filter is then amplified by about a factor of 1000 and fed to the RF input of a second mixer (M2). The LO input of the second mixer is connected to the output of the second synthesizer, which provides a frequency exactly 40Hz below this intermediate frequency. After bandpass filtering and amplification, the output of M2 contains all the information of the original high frequency signal. The reference channel operates in a similar fashion.

The amplification of the signal at 100KHz, second stage mixing, and further amplification and low pass filtering is obtained using a Standard Research systems (model SR530), lock-in amplifier, equipped with a tunable input filter set at 100KHz. The low frequency filtering at 40Hz and amplification is performed using standard digital cross-correlation electronics. At this point, four signals, corresponding to the AC and DC of the sample and of the reference, have been generated. These signals are applied to the four inputs of the analog converter interface card for a PC computer, where the usual acquisition and processing are performed under computer control.

The electronic circuit has a flat frequency response to about 1GHz, is highly sensitive, andis immune from synthesizer phase noise. Preliminary tests have shown that the phase can be measured with an accuracy of about 0.2° with a 100s integration time up to 4GHz using a microchannel plate detector. The ultimate time resolution using phase measurements with the microchannel plates is about 100fs, which corresponds to a phase delay of 0.2° at 1GHz. Of course, other factors may limit the maximum resolution.

IV. PRELIMINARY RESULTS

Preliminary measurements on a solution of NATA (N-acetyl-tryptophanamide) in water were obtained using the zero order of the Al-Seya monochromator at SRC. The light was quasi-monochromatized using an interference filter centered at 295nm with a 10 nm bandpass. In the first run, the ring operated in the 15-bunch mode, which gives a minimum frequency of 50.582 MHz. The results for the phase and the modulation are shown in Fig. 6a by circles and squares, respectively. Over a period of hours, the phase and modulation stability were superb. The natural decay of the beam intensity did not modify the modulation ratio or the phase value.

For a complete lifetime frequency measurement, it is important to have access to high and low frequencies. In the next injection, the intensity of one bunch was decreased by about 20% and the master oscillator was shifted to 50.5821 MHz (an integer multiple of 15). Under this condition, harmonic frequencies of the 3.3772 MHz single-bunch fundamental were obtained as shown in Fig. 6b. The solid and dotted lines drawn in the figure were obtained using Eqs. 3 and 4 and the well known lifetime of NATA, 3 ns. The residues plot showed an exceptionally small deviation as compared with similar experiments using a laser source.

V. PLANNED RESEARCH

The fluorescence beamline at Aladdin will service the national biochemical and biophysical research community, both industrial and academic. Fluorescence spectroscopy is both exquisitely sensitive and widely versatile. Its dynamic range for detection ranges from near 10 pM to the millimolar range. Moreover, the use of extrinsic probes and multiple fluorescence observables, color, intensity and polarization, renders this technique ideal for monitoring the complex interactions between biological macromolecules and for

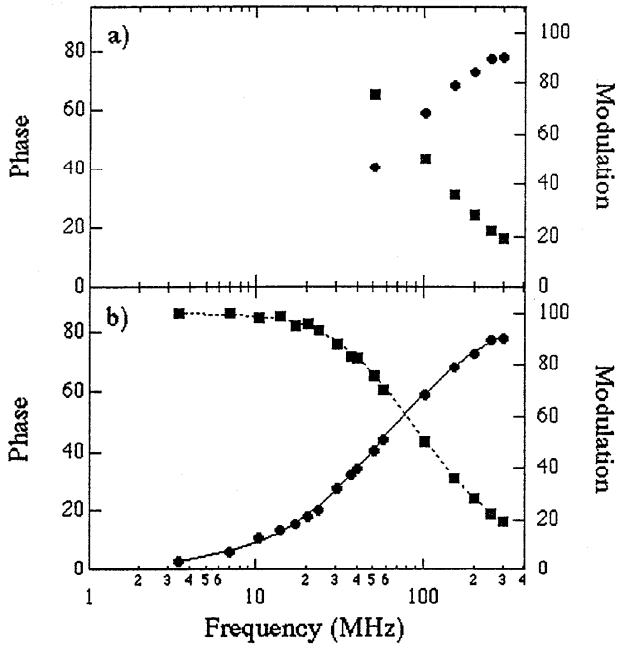

FIG. 6. Phase (circles) and modulation (squares) as a function of the frequency obtained on a solution of NATA (N-acetyl-tryptophanamide) in water. (a) Aladdin operating in its normal mode. (b) The intensity of one of the 15 bunches in Aladdin is 20% that of the rest. Solid and dotted lines, fits with τ=3 ns to the phase and modulation using Eqs. 3 and 4, respectively.

characterizing the multiple conformational substates these biopolymers adopt. The ability to time resolve the various fluorescence observables and to study association and conformational phenomena at equilibrium and kinetically on the millisecond-to-second time scale allows for the determination of the affinity constants and rate constants associated with biopolymer interactions. The Aladdin light source with its continuously variable wavelength over the entire UV-VIS range, and its stable and narrow pulse characteristics make it ideally suited for sophisticated fluorescence spectroscopic analysis of macromolecular complexes.

Many of the systems that will be studied at Aladdin represent important steps or control points in cellular function, differentiation and growth. Obviously the understanding of the fundamental physical mechanisms for control of these functions is required for the eventual development of therapeutic strategies to correct the malfunctions in these systems that lead to diseases, such as cancer and immune diseases. Primary systems to be studied at Aladdin include a group of eukaryotic transcriptional regulatory proteins and the nuclear receptor proteins, specifically, studies of the interactions between the glucocorticoid, estrogen, vitamin D and retinoic acid receptors with their target DNA response elements. The stoichiometries, cooperativities, and relative affinities of these complexes will be contrasted with the goal of characterizing the basis for biological specificity in these systems. These studies will be based on the use of fluorescently labeled oligonucleotides and proteins. Anisotropy changes will reflect changes in complex size and hence binding. Energy transfer between labeled proteins and labeled DNA or between labeled heterologous proteins on unlabeled DNA will allow for distance mapping of the various components of these macromolecular complexes. Moreover, although these distance maps do not provide the resolution of x-ray or NMR techniques, they have the advantage that they can be carried out over a wide range of concentration, solution conditions and temperature, thus allowing for the observation of changes in the complex structure with changing environment. Eventually, we foresee the coupling of a near-field optical microscope to the beamline for single molecule detection of fluorescently labeled protein-DNA complexes.

A great deal of effort will be expended using the multi-wavelength characteristics of the Aladdin light source to develop and characterize the most suitable fluorescent dye conjugates of proteins and nucleic acids. Effects of changes in the electrostatic environment of the nucleic acid, sequence length and double- or single-stranded characteristics will be evaluated. Various methods and chemistries of dye coupling and multiple dyes will be characterized for their response to complexation. Changes in color, intensity, lifetime, and steady-state and time-resolved anisotropy will be measured. Moreover, the sensitivity limits will remain a primary concern. The use of labeled oligonucleotides for the detection of protein complexation and hybridization is in its infancy. The results of this development project will have far-reaching consequences in the implementation of nonradioactive assays in the research environment and also in the pharmaceutical industry and the clinic.

While the protein-DNA complexation project will be the main focus of the research of the coordinating team, composed of the first four authors and their coworkers, the fluorescence beamline at Aladdin will provide service for the biochemical and biophysical community at large. Examples of projects to be performed at Aladdin include a cooperative effort on cell/cytokine recognition between one of us (CAR) and researchers at Genentech in South San Francisco. Protein folding and structural dynamics will also be studied by an Italian research group. On the UW campus, a number of researchers in chemistry and the biological sciences have indicated interest in the use of the fluorescence beamline. A researcher from the Chemistry Department will use the Aladdin beamline to probe polymer dynamics near the glass transition. The Laboratory for Fluorescence Dynamics, a National Fluorescence facility in Urbana, Illinois, with dozens of visitors each year, will route users to the fluorescence beamline at Aladdin in cases where the LFD cannot provide the appropriate excitation wavelength or alternately if the user schedule is booked. Fluorescence spectroscopy is becoming more and more popular as its ease of use increases and the tools for global analysis of multidimensional data surfaces have become available.

VI. DISCUSSION

The optical design, principle of operation, and the near-future scientific program of a new UV-VIS beamline for phase fluorometry at SRC have been presented. Preliminary results have demonstrated the excellent phase and modulation stability as well as speed of data collection. The system measures lifetimes as short as picoseconds. The intensity reduction of one of the bunches, practically unnoticed by the rest of the users, extends the measurable lifetimes to almost one microsecond.

In addition to the beamline, a biochemistry laboratory for biological sample preparation, purification, testing of activity, manipulation (e.g., membrane reconstitution) and verification of sample integrity post-fluorescence experiment is almost complete at the SRC site.

ACKNOWLEDGMENTS

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