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PLASTIQUE: A SYNCHROTRON RADIATION BEAMLINE FOR TIME RESOLVED FLUORESCENCE IN THE FREQUENCY DOMAIN

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ABSTRACT

PLASTIQUE is the only synchrotron radiation beamline in the world that performs time resolved fluorescence experiments in frequency domain. These experiments are extremely valuable sources of information on the structure and dynamics of molecules. We describe the beamline and some examples of initial data.

1. - A BEAMLINE FOR FLUORESCENCE

Time-resolved fluorescence is one of the leading techniques in molecular dynamics. Only in recent years, however, this technique has exploited the unique characteristics of synchrotron radiation. We describe PLASTIQUE, the only operating synchrotron radiation beamline for frequency-domain fluorometry. In particular, PLASTIQUE is the only fluorescence facility of its kind for near UV photons, with a total range of 200-800 nm (see Fig. 1). No tunable conventional sources exist for the lower part of this spectrum.

A pulsed light source as provided by synchrotron radiation is uniquely suited for excitation in time-resolved fluorescence, because of the possibility of continuously varying the wavelength range, and because of the short duration and high repetition rate of the pulse. Generally, the fluorescence emission after pulse excitation is measured in the time domain, using the popular technique of the time-correlated single-photon counting (SPC). Phase fluorometry, however, can also be used in conjunction with a high repetition rate pulsed light source, with the advantages of the armonic method. These advantages include the high accuracy

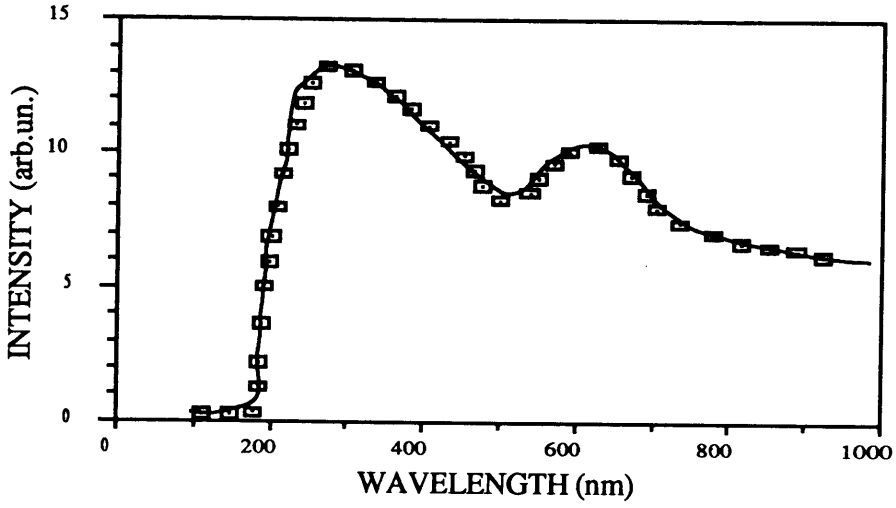

FIG. 1 - The intensity spectrum of PLASTIQUE. The low wavelength limit is determined by the cutoff of the fused silica window.

Ordinarily, multifrequency phase fluorometry is implemented with sinusoidally modulated sources.[1] The time structure of synchrotron radiation simultaneously provides a large number of modulation frequencies. PLASTIQUE is connected to the storage ring ADONE at the Frascati National Laboratory, whose single-bunch Gaussian light pulse contains a set of harmonic frequencies 2.856167 MHz apart ("comb function"), with a Gaussian-envelope half width of 500 MHz.[2] A pioneering work by Weber *et al.* made it possible to extract a single harmonic frequency with a powerful technique: the cross-correlation method.[3]

The wide emission spectrum of synchrotron radiation provides a tunable excitation light source. This opens the way to direct differential measurements of fluorescence excited by different wavelengths. With PLASTIQUE, these measurements are implemented in a wide range, 200-800 nm; neither tunable lasers nor lamps exist in the 200-280 nm portion of the range. PLASTIQUE is used by a wide variety of users from Italy and the USA, for experiments in diverse areas of the life sciences and materials science.

2 - TECHNICAL DESCRIPTION

PLASTIQUE collects synchrotron radiation emitted by a bending magnet on the 1.5 GeV storage ring ADONE, over 2.5 mrad on the horizontal plane, and 4 mrad on the vertical plane. Figure 2 shows an overall view of the beamline. The radiation is deflected and separated from the x-ray beam of an adjacent beamline by means of a 2° incidence plane mirror (S3 in Fig. 2) at 11 m from the source. A fused silica window separates the ultra high vacuum ($p=10^{-9}$ mbar) section connected to the storage ring from the low vacuum ($p=10^{-3}$ mbar) beamline.

A spherical focussing mirror (M1), with a 25.0 m curvature radius, deflects the beam upward by a 105° angle. Another plane mirror (M2) restores the horizontal direction. A second fused silica window separates the low vacuum chamber from the atmosphere. After this window, therefore, the beam travels in air. The light is focussed by a cylindrical silica lens to the entrance slit of a Jobin Yvon H10 100 mm spherical grating monochromator, followed by the experimental apparatus. In the final stage of this apparatus the beam is focussed again by a spherical silica lens. This lens focuses the monochromatic light on the sample, where the spot

size is approximately 1 mm, as shown in Fig. 3 on millimeter paper, in very good agreement with the ray tracing simulation performed during the project phase of the beamline.

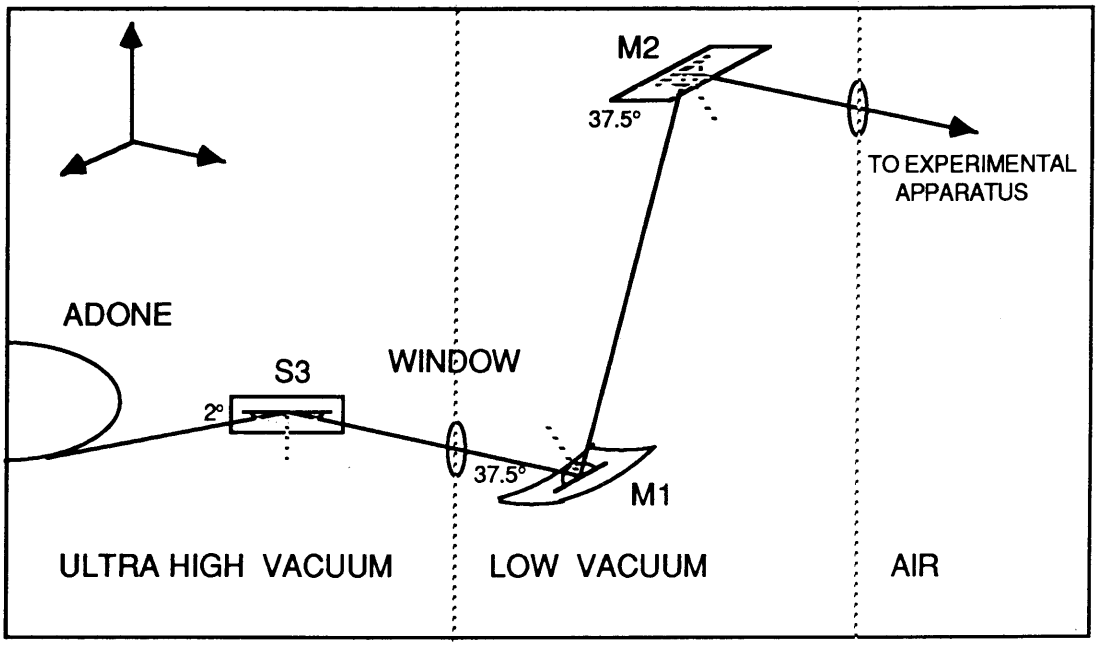

FIG. 2 - The layout of PLASTIQUE on the ADONE storage ring. Note the three sections: ultra-high vacuum, low vacuum and air pressure.

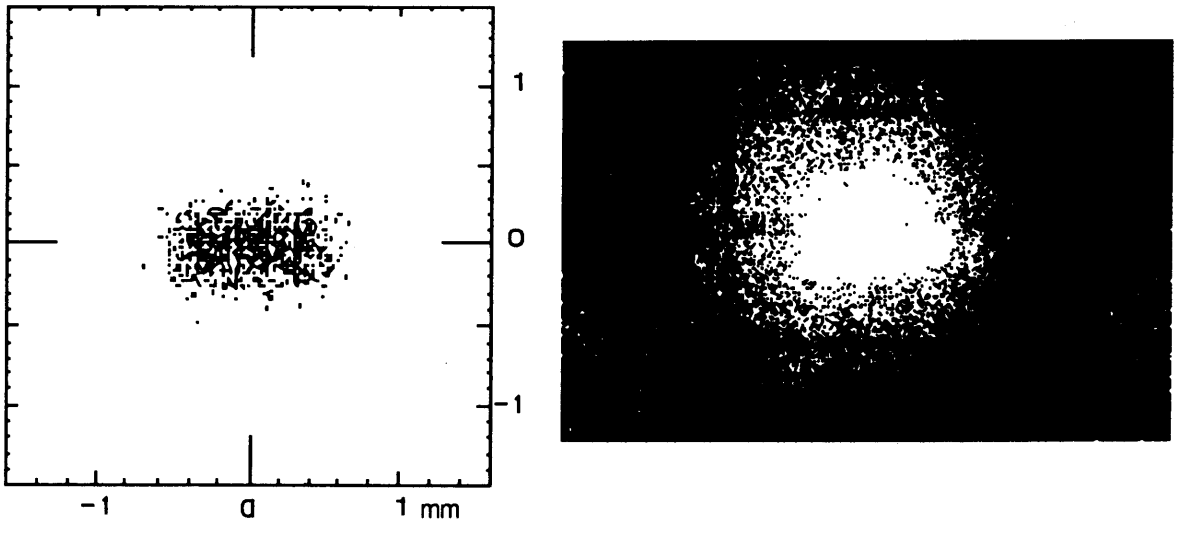

FIG. 3 - The image of synchrotron radiation beam, simulated by the ray tracing program SHADOW (F. Cerrina). The image shows 1000 rays focussed on the sample (left) and a photograph of the beam (right) in the same place on millimeter paper.

2.1 - LOW VACUUM SECTION

We do not need ultra high vacuum, because light in our spectral range does not interfere significantly with air, and a low vacuum is sufficient to maintain the mirror surfaces clean. The high-energy cutoff is determined by the insertion of silica windows (7.75 eV). The low vacuum section is completely built using PVC pipes, inspirating the name of the beamline.[4] Vacuum tests were made on several kinds of plastics. Low and high density polyethylene did not show

section is completely built using PVC pipes, inspirating the name of the beamline.[4] Vacuum tests were made on several kinds of plastics. Low and high density polyethylene did not show enough mechanical stiffness. Poly-carbonate was mechanically adequate, but large pipes were not readily available (we needed a diameter > 70 mm). PVC (poly-vynilchloride) was chosen because it satisfies all mechanical requirements, and it is capable of maintaining a 10^{-3} mbar pressure. Furthermore PVC is particularly simple to machine and very light, making it easy to sustain the vacuum pipe from the laboratory's ceiling.

2.2 - MIRRORS

All the three mirrors are coated by aluminum, because this metal has high reflectivity in the UV spectral range; a magnesium fluoride overcoating, transparent in the range of interest, protects the Al coating from oxidation. The plane mirror S3 is not accessible when ADONE is operating because it is inside the radiation shielding wall; the plane mirror M2 is not reachable because it is approximately 5 m above the floor level. The two mirrors, therefore, must be moved by remote control from the experimental station.

3 - EXPERIMENTAL TECHNIQUE

A spherical grating monochromator (Jobin-Yvon H10 equipped with 1200 lines/mm grating, focal length 10 cm, f=3.5) sends the beam to an optical module (SLM OP450) equipped with a rotating turret to permit easy exchange between sample and reference. This turret is moved by a synchronous motor. Liquids in standard fluorescence cuvettes as well as solid samples can be analyzed. For liquid samples, a circulating thermostatic bath can be used to control the temperature. The sample emission is collected by a large aperture lens and focused onto a photomultiplier PMT2 (Hamamatsu R928). A quartz beam splitter is placed in the optical path before the sample, to direct a fraction of the exciting light to a reference photomultiplier (PMT1), that measures the intensity and phase of the excitation signal (Fig. 4).

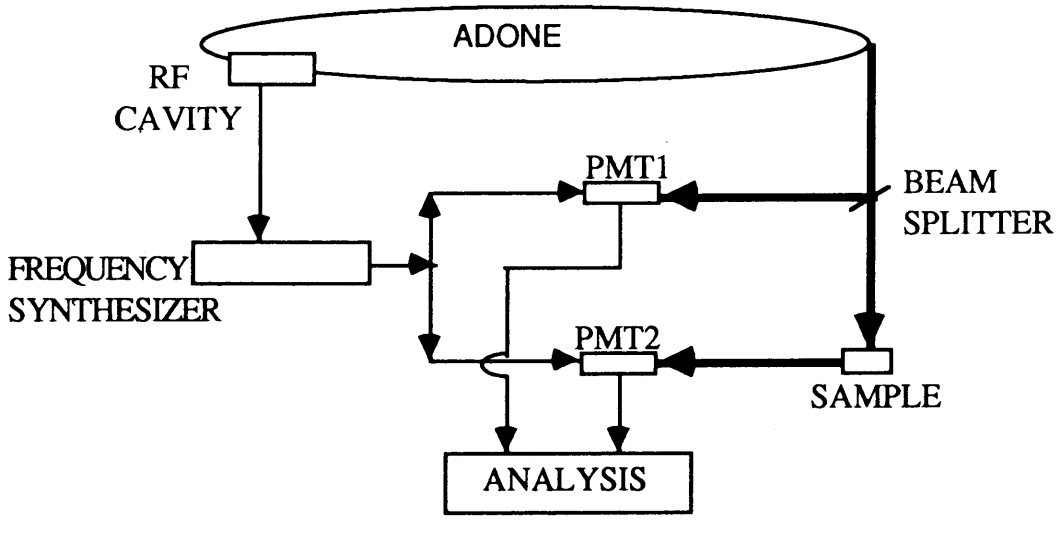

FIG. 4 - The block diagram of the multifrequency phase and modulation fluorometer.

A slave frequency synthesizer (Marconi Instruments 10 kHz - 1 GHz signal generator 2022A) is in phase with the synthesizer that drives the RF cavity of the storage ring, thus with the light pulse repetition frequency.

Note that the *slave* frequency synthesizer takes, as a reference signal, the output of the synthesizer that drives the RF cavity. This output is extremely stable and reproducible, about one order of magnitude better than the synthesizers usually used on conventional phase fluorometers, or in the first synchrotron radiation fluorometer built in Frascati in 1983 [2]. It results in ten times more accurate phase measurements, or in ten times shorter acquisition time.

The output of the slave synthesizer can be varied in order to obtain a frequency equal to ADONE's fundamental, or to one of the harmonic components, plus 40 Hz. The small difference, 40 Hz, is the cross-correlation frequency. The output of the synthesizer is amplified by an RF power amplifier (ENI 503L), splitted in two equal parts and applied to the second dynode of the PMTs to implement the cross-correlation technique. We can now perform experiments up to about 330 MHz, even if in principle it is possible to push data acquisition up to the limits of the frequency synthesizer (1GHz). The actual limit is imposed by the photomultipliers maximum operational frequency.

The outputs of the two PMTs are analyzed separately by two identical channels (CH1 and CH2) in the analysis unit (ISS GREG 80). The two signals are amplified and separated into DC and AC components. The DC component is integrated, to generate a DC signal proportional to the average intensity of the detected signal. The AC component is sent to an amplifier and filtered by a band pass active filter to select the 40 Hz component. The output of the filter is rectified and integrated to produce a continuous voltage proportional to the AC component. The DC and AC parts of each channel are continuously monitored by four digital voltmeters, not used for data acquisition, but only for rapid inspection of the signal levels. Accurate measurements of the signals are performed by a precision 13 bits integrating-digital-voltmeter with 0.1 mV resolution.

The output of the electronics described before is interfaced with an IBM PS 30 computer, that for each frequency calculates the demodulation ratio M

$$M = \frac{AC_{em} / DC_{em}}{AC_{ex} / DC_{ex}} \qquad (1)$$

The phase difference between the reference and the sample signal ϕ is measured by a digital phasemeter. The output of two active filters (CH1 and 2) are sent to zero-crossing detectors, where two square-waves are produced. The positive square waves are used to start and stop the phase counter in the computer interface. The resolution for phase measurements is 1 microsecond, which corresponds to an angular resolution of about 0.01°.

From the two simple equations

$$\phi = \tan \omega t_{\phi} \qquad (2)$$

and

$$M = (1+\omega^2 \tau_M^2)^{-1/2} \qquad (3)$$

4. EXAMPLES OF EXPERIMENTAL DATA

PLASTIQUE has already been used for a variety of tests and experiments demonstrating that samples with 0.1 absorbance are crearly measurable. We present here two specific examples, Fig. 5 and 6. In Fig. 5, we show phase and modulation data as a function of frequency for a solution of popop in ethanol. The reference was a scattering solution of glycogen in water. The excitation wavelength was 340 nm, and the sample emission light was filtered by a cutoff filter at 390 nm, to eliminate the Raman scattering signal from water. Data were acquired over a frequency range from 8.6-154.2 MHz. A least-square analysis of these data gives a single lifetime component (monoexponential decay) of 1.300 ns, close to the expected value (this is a standard fluorophore), and $\chi 2=1.5$ ($\chi 2=\Sigma_\omega(\phi_c-\phi_m)^2 + +\Sigma_\omega(M_c-M_m)^2$, where ϕ_m is the measured phase at a given frequency ω , ϕ_c is the calculated phase during each iteration step of the minimization performed by the SIMPLEX routine. And M_m and M_c are the measured and calculated modulation ratios.

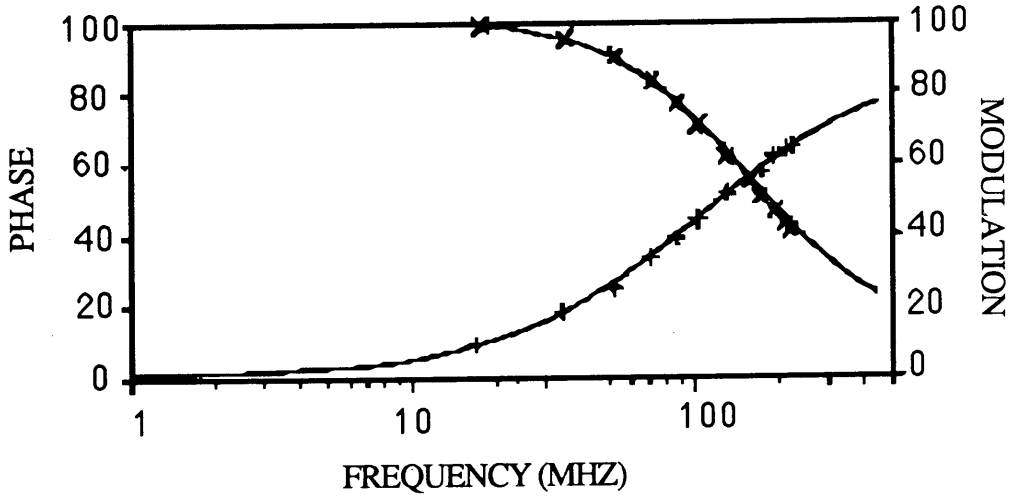

FIG. 5 - Phase (+) and modulation (x) vs light modulation frequency data for POPOP in ethanol.

Note the excellent correspondence between the experimental data and the solid lines, calculated for a lifetime of 1.290 ns.

Fig. 6 shows phase and modulation data for a solution of a dimeric protein (human copper-zinc superoxide dismutase) containing two equivalent tryptophans.[5] Excitation was at several wavelengths ranging from 260-290 nm, with a bandwidth of 8 nm.

The absorbance of the sample at 280 nm was about 0.2. Fluorescence was collected after passing through a 320 nm cutoff filter to remove the scattered light. Fluorescence experiments were performed at 25°C. The data were analyzed either with a single and double exponential decay or with a model based on a continuous distribution of lifetime having a Lorentzian shape. [5] In this latter case, the center and the width of the Lorentzian function were obtained by minimizing the $\chi 2$ values with a routine based on the SIMPLEX method.

This procedure demonstrated that the fluorescence decay cannot be described with a single lifetime, although the two tryptophans contained in the dimer are fully equivalent and both exposed to the solvent. A Lorentzian continuous lifetime distribution (centered at 2.2 ns and with full width at half maximum of 0.3 ns) is needed to obtain a good fit of the data. [5]

Note that the excitation wavelength range that we used in this experiment was not available anywhere else, and this experiment could only be performed on PLASTIQUE apparatus.

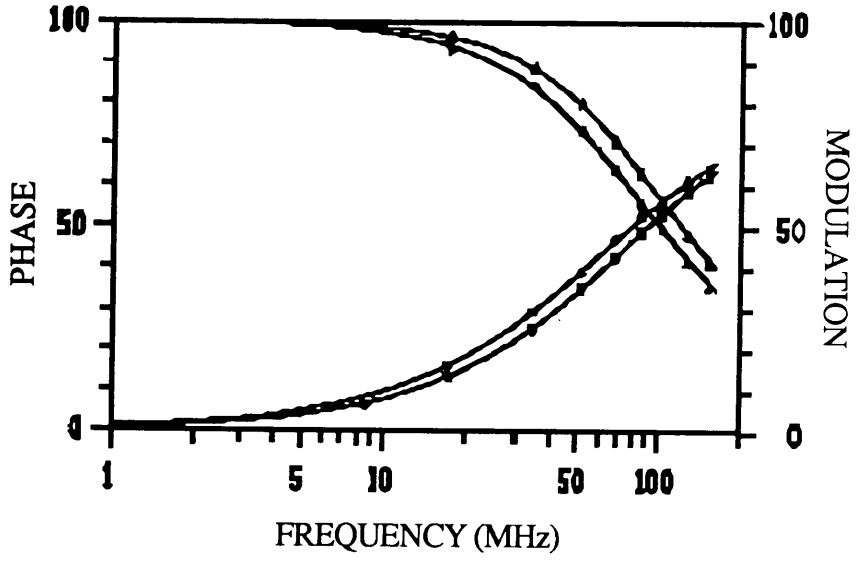

FIG. 6 - Phase (circles) and modulation (triangles) values as a function of light modulation frequency, for holo (full symbols) and apo (open symbols) human copper-zinc superoxide dismutase. the solid lines correspond to the best fits.

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4) The word PLASTIQUE means indeed "plastic" in French. However, the name of the beamline was also interpreted as an acronym in Italian, for "Picclola Linea Accroccata Senza Tanta Immaginazione, Qualitativamente Un Errore", that means "small beamline put together without much imagination, a qualitative mistake".

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